Hsp60 accelerates the maturation of pro-caspase-3 by upstream activator proteases during apoptosis

Steven Xanthoudakis1, Sophie Roy, Dita Rasper, Trevor Hennessey, Yves Aubin, Robin Cassady, Paul Tava, Rejean Ruel, Antony Rosen2 and Donald W. Nicholson

Department of Biochemistry and Molecular Biology, Merck Frosst Centre for Therapeutic Research, Kirkland, Quebec, Canada H9H 3L1 and *Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA
1Corresponding author
e-mail: steven_xanthoudakis@merck.com

The activation of caspases represents a critical step in the pathways leading to the biochemical and morphological changes that underlie apoptosis. Multiple pathways leading to caspase activation appear to exist and vary depending on the death-inducing stimulus. We demonstrate that the activation of caspase-3, in Jurkat cells stimulated to undergo apoptosis by a Fas-independent pathway, is catalyzed by caspase-6. Caspase-6 was found to co-purify with caspase-3 as part of a multiprotein activation complex from extracts of camptothecin-treated Jurkat cells. A biochemical analysis of the protein constituents of the activation complex showed that Hsp60 was also present. Furthermore, an interaction between Hsp60 and caspase-3 could be demonstrated by co-immunoprecipitation experiments using HeLa as well as Jurkat cell extracts. Using a reconstituted in vitro system, Hsp60 was able to substantially accelerate the maturation of pro-caspase-3 by different upstream activator caspases and this effect was dependent on ATP hydrolysis. We propose that the ATP-dependent ‘foldase’ activity of Hsp60 improves the vulnerability of pro-caspase-3 to proteolytic maturation by upstream caspases and that this represents an important regulatory event in apoptotic cell death.

Keywords: apoptosis/caspase/chaperonin/Hsp60/mitochondria

Introduction

Apoptosis is an evolutionarily conserved mode of cell death characterized by a discrete set of biochemical and morphological events which result in the ordered disassembly of the cell (Kerr et al., 1972; Vaux et al., 1994; Steller, 1995). Among the key cellular apoptotic mediators are a group of conserved cysteinyl-aspartate proteases that are activated in response to a variety of proapoptotic stimuli (Nicholson and Thornberry, 1997; Thornberry et al., 1997b; Thornberry and Lazebnik, 1998). In humans, these proteases, collectively termed caspases, comprise a multigene family of at least 10 members encoded by distinct genes (Alnemri, 1997; Thornberry, 1997). The caspases are normally expressed as latent zymogens, that in response to an apoptotic stimulus are proteolytically cleaved at specific Asp-X junctions to generate catalytically active heterodimers. Based on crystallographic analysis, the mature enzyme consists of two large (~p20) and two small (~p10) subunits, derived from a pair of interdigitated proenzyme molecules (Rota, et al., 1996). Following activation, caspases have been shown to cleave, in a sequence-specific manner, a variety of cellular proteins that mediate diverse functions (Casola-Rosen et al., 1996; Thornberry and Lazebnik, 1998). Biochemical studies using synthetic peptide substrate libraries have established the substrate specificity of each of the caspase family members (Thornberry et al., 1997a). On the basis of these studies, the caspases segregate into three sub-groups that also reflect their functional differences: (i) the group I caspases (caspases-1, -4 and -5) which are involved mainly in mediating proinflammatory responses; (ii) the group II caspases (caspases-2, -3 and -7) that act primarily as ‘effector’ proteases and which, during apoptosis, recognize and cleave sequence motifs of the type DEXD found in a number of known proteolytic targets; and (iii) the group III caspases (caspases-6, -8, -9 and -10), which recognize the sequence motif (IVL)EXD and function principally to catalyze the activation of the group II caspases. With regard to the latter sub-group, the molecular ordering of group III (activator) caspases upstream of the group II (effector) caspases has been demonstrated in both non-receptor- and receptor-mediated signaling pathways. For example, in the Fas–CD95 system, binding of Fas ligand to its receptor results in oligomerization and activation of caspase-8 through a well-defined death receptor complex (Boldin et al., 1996; Muzio et al., 1996; Medema et al., 1997; Scaffidi et al., 1997; Vincenz and Dixit, 1997). Once activated, caspase-8 in turn can act upon caspase-3 and/or caspase-7 to catalyze their activation (Orth et al., 1996; Srinivasula et al., 1996; Juan et al., 1997). A similar caspase cascade has been implicated in signaling via the tumor necrosis factor (TNF) family of death receptors (Wallach et al., 1997, 1998; Ashkenazi and Dixit, 1998).

At the moment, less is known about the non-receptor signal transduction pathways that operate within the cytoplasm to activate caspases. At least one apoptotic pathway in mammalian cells is shunted through the mitochondria and involves the export of cytochrome c to the cytoplasm (Liu et al., 1996b). The release of cytochrome c from mitochondria is associated with the activation of a multimeric complex comprising cytochrome c, caspase-9 and Apaf-1, the mammalian counterpart of the nematode CED-4 protein (Li et al., 1997; Vaux, 1997; Zou et al., 1997). Caspase-9 and Apaf-1 are believed to interact via a caspase recruitment domain (CARD) located at the N-terminus of either protein (Chou et al., 1998). Cytochrome
c-dependent activation of the complex results in the maturation of caspase-9, which subsequently cleaves and activates caspase-3. The arrangement of proteins along this pathway is supported by recent knockout studies which demonstrate that caspase-3, caspase-9 and Apaf-1 null mice exhibit similar phenotypes, albeit with different severities (Kuida et al., 1996; Cecconi et al., 1998; Kuida et al., 1998; Yoshida et al., 1998).

The notion that mitochondria act as a 'sensor' for transducing apoptotic stimuli is supported further by studies showing localization of caspase-3 as well as members of the Bcl family of proteins to this organelle (Green and Reed, 1998; Mancini et al., 1998). The mitochondrial pool of caspase-3 is normally latent, but is redistributed into the cytoplasm following activation in response to UV irradiation (Mancini et al., 1998). Since the activation of caspase-3 via the cytochrome c–Apaf-1–caspase-9 pathway is thought to be an extra-mitochondrial event (Li et al., 1997; Zou et al., 1997), it is likely that additional mechanisms would act to process caspase-3 depending on the subcellular localization of the latent proenzyme. Furthermore, the mechanism of caspase-3 activation is also likely to be stimulus as well as cell type specific. In the present study, we sought to identify the activator protease that is responsible for proteolytic activation of caspase-3 in Jurkat cells following treatment with camptothecin, a potent topoisomerase inhibitor (Hsiang et al., 1985). Through chromatographic purification, we identify the activator protease to be caspase-6. Interestingly, caspase-3 co-purifies with caspase-6 as part of a multiprotein complex which also contains Hsp60, a mitochondrial chaperonin. In addition, we show that Hsp60 promotes the activation of pro-caspase-3 by upstream caspases.

Results

Purification of the caspase-3 maturase

The purification strategy for identifying a protease, presumably an upstream activator caspase that could catalyze the maturation of pro-caspase-3 in apoptotic cells, was analogous to the strategy used in the original identification of caspase-3 (Nicholson et al., 1995). Purification of the maturase activity involved ion-exchange fractionation followed by affinity chromatography. In all fractionation steps, protein elution was monitored by continuous UV (280 nm) absorption. Caspase activity was analyzed by monitoring cleavage of a fluorogenic tetrapeptide substrate (IETD-AMC) corresponding to the cleavage motif located at the junction between the large (p17) and small (p12) subunits of pro-caspase-3. As a first step in the purification, ~450 mg of crude Jurkat whole-cell extract from camptothecin-treated cells was applied to a 53 ml Hi-load Q-Sepharose column and eluted using a linear NaCl gradient (Figure 1A). IETD-AMC cleavage activity eluted as two distinct peaks at 0.1 and 0.4 M NaCl (Figure 1B). A preliminary investigation of the chromatographic properties of the IETD-AMC cleavage activity from control versus camptothecin- or anti-Fas-treated cells revealed that the first peak of IETD-AMC cleavage activity was unique to extracts from apoptotic cells (Figure 2). This peak comprised a mixture of protease activities capable of processing both radiolabeled pro-caspase-3 and poly(ADP-ribose) polymerase (PARP), a well characterized caspase-3 substrate (Laizemik et al., 1994; Nicholson et al., 1995; Tewari et al., 1995) (Figure 1B and C). Furthermore,
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Fig. 2. Comparison of caspase-3 maturase activity in Jurkat cells following treatment with different apoptotic stimuli. Extracts were prepared from control (untreated), αFas- and camptothecin-treated Jurkat cells and fractionated by Hi-load Q-Sepharose chromatography. The profile of IETD-AMC cleavage activity from the fractionation is shown. Cleavage of IETD-AMC was measured by fluorometry. Note that the first peak of activity detected in extracts from αFas- and camptothecin-treated cells is absent from the control extracts and is specific to apoptotic cells.

Pro-caspase-3 was detected in this peak by immunoblot analysis. Following incubation at 37°C, the pro-caspase-3 detected could be converted to its mature form in a time-dependent manner (Figure 1D). The second peak of activity, which eluted at a higher salt concentration (0.4 M), was found in extracts from apoptotic as well as control cells (Figure 2). This peak comprised a non-specific IETD-AMC degradative activity which failed to process either pro-caspase-3 or PARP (Figure 1C).

Selective enrichment of the caspase-3 maturase was achieved through affinity chromatography using a biotinylated tetrapeptide aldehyde ligand resembling the activation site within pro-caspase-3 (biotin-IETD-CHO) (Nicholson et al., 1995). Fractions eluting with the first peak of IETD-AMC cleavage activity from the ion-exchange column were pooled and diluted to decrease the concentration of salt. The biotinylated affinity ligand was pre-incubated with the diluted activity to allow the inhibitor to equilibrate with the enzyme before being harvested on a streptavidin–agarose resin. Once bound with the IETD cleavage activity, the affinity column was washed stringently (0.5 M NaCl) to remove proteins that were associated non-specifically with the resin. Elution of the IETD cleavage activity was achieved in two stages by competitive displacement using a non-biotinylated tetrapeptide aldehyde inhibitor (IETD-CHO). The first elution step was carried out immediately following the wash, and the second after an overnight incubation in the presence of the inhibitor. This extended elution protocol was used to overcome the slow off-rate of the aldehyde inhibitor.

The presence of inhibitor in the eluted fractions precluded analysis of enzymatic activity using the fluorogenic assay. The profile of eluted polypeptides was therefore assessed by SDS–PAGE. Only the eluate from the overnight co-incubation with the inhibitor yielded any detectable protein as determined by silver staining analysis of the gel. As shown in Figure 3B, the peak protein fraction contained a series of polypeptides with molecular masses ranging from 12 to 116 kDa. Two of the polypeptides detected, with molecular masses of ~12 and 17 kDa,
migrated with a mass that was characteristic of mature caspase subunits. To investigate this possibility, immunoblot analysis was performed using a panel of antisera directed against caspases 1–9. The Western blot revealed that caspases-3 and -6 were indeed present in the affinity-purified material (Figure 3C). This result identifies caspase-6 as the IETD cleavage activity in extracts from camptothecin-treated Jurkat cells. These findings are consistent with its substrate specificity and its designation, on the basis of biochemical analysis, as a group III activator caspase (Liu et al., 1996a; Thornberry et al., 1997a).

Characterization of caspase-associated proteins

In addition to the caspases, several higher molecular weight proteins were detected in the affinity-purified fractions that failed to cross-react with any of the caspase antibodies tested (Figure 3B). We refer to these polypeptides as CAPs. To determine the identity of the 58–60 kDa CAPs, the proteins were gel extracted, subjected to tryptic digestion and resolved as individual peptides by HPLC. Two distinct peptide fragments were analyzed by tandem mass spectrometry, both of which generated sequence information that was unambiguous. Using either sequence, a search of the DDBJ/EMBL/GenBank database revealed the 58–60 kDa CAP to be heat shock protein 60 (Hsp60). The identity of Hsp60 was confirmed by immunoblot analysis of the affinity-purified protein using a polyclonal antibody raised against human Hsp60 (Figure 3C).

To examine further the interaction between caspase-3, caspase-6 and Hsp60, immunoprecipitation experiments were performed using polyclonal antibodies directed against the caspases (Figure 4A). Both anti-caspase-3 and anti-caspase-6 antisera were capable of immunoprecipitating Hsp60 from the ion-exchange chromatography fractions enriched with IETD cleavage activity (Figure 1B). A similar result was obtained with metabolically labeled HeLa cell extracts using the anti-caspase-3 antisera (Figure 4B). Hsp60 failed to co-immunoprecipitate using rabbit pre-immune serum or anti-caspase antibodies directed against other effector caspases including caspase-2 and caspase-7 (data not shown). In addition, co-immunoprecipitation of Hsp60 with caspase-3 was precluded when HeLa cell lysates were prepared in the presence of SDS and then diluted prior to the addition of caspase-3 antisera (data not shown). It is noteworthy that in these studies caspase-3 co-immunoprecipitated with Hsp60 as a 32 kDa proenzyme. Pro-caspase-3 was also the predominant species detected after ion-exchange chromatography, suggesting that Hsp60 recognizes and may associate initially with the latent form of caspase-3.

Hsp60 promotes activation of pro-caspase-3

To assess the functional significance of the interaction between the caspases and Hsp60, we prepared recombinant pro-caspase-3 as a fusion protein harboring an N-terminal polyhistidine tag to facilitate purification. Experiments were designed to evaluate the influence of Hsp60 on activation of the proenzyme and to determine whether any observed effect was direct, or mediated through an activator protease. Reaction mixtures were reconstituted using purified protein components, and activation of pro-caspase-3 was measured by monitoring cleavage of the fluorogenic caspase-3 substrate (DEVD-AMC). As shown in Figure 5A, activation of caspase-3 by caspases-6, -8 and -9 was markedly enhanced in the presence of recombinant Hsp60. A titration of Hsp60 on caspase-6-mediated activation of caspase-3 showed this effect to be dose dependent (Figure 5A, inset). This stimulatory effect by Hsp60 was not manifested in the absence of an activator caspase, indicating that the interaction between Hsp60 and pro-caspase-3 is not in and of itself sufficient to mediate activation. Processing of caspase-3 by the activator caspases was confirmed by immunoblot using antibodies directed against the large subunit of caspase-3 (Figure 5B). The data suggest that Hsp60-mediated stimulation of caspase-3 proteolytic maturation occurs via an upstream activator protease, perhaps through stabilization of a ternary complex. In cells, Hsp60 functions as a multimeric ring complex consisting of 14 subunits (Beissinger and Buchner, 1998). A titration of Hsp60 on caspase-6-mediated activation of caspase-3 suggested that the multimeric form of Hsp60 may promote activation of the proenzyme. This is based on the observation that a 16-fold molar excess of Hsp60 monomer stimulated complete maturation of caspase-3, whereas an equimolar amount of Hsp60 was inefficient at stimulating maturation (Figure 5A, inset). Regardless of the mechanism, the stimulatory effect of Hsp60 appears to have an energy requirement, as the addition of exogenous ATP further stimulated caspase-3 activation, albeit modestly. In the absence of Hsp60, ATP alone had no effect on caspase activation. In addition, a non-hydrolyzable analog of ATP (ATPγS), when preincubated with Hsp60, inhibited Hsp60-mediated maturation of pro-caspase-3 by caspases-6, -8 and -9 (Figure 5A and B). This inhibitory effect was only partially attenuated when ATPγS was added to the reaction mixture following the initial incubation between Hsp60 and pro-caspase-3, suggesting that subsequent activation by an activator caspase is largely dependent on a persistent interaction.
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Fig. 5. Hsp60 facilitates maturation of caspase-3 by activator caspases. (A) Pro-caspase-3 (0.04 μM) was pre-incubated for 10 min with Hsp60, ATP, ATPγS, buffer (as indicated) (0.66 μM) prior to the addition of buffer or caspases-6, -8 or -9 (0.08 μM). Incubation of Hsp60 with ATP or ATPγS was carried out for an additional 10 min at room temperature prior to the incubation with pro-caspase-3. Upon addition of caspases-6, -8 and -9, the reactions were incubated for 2 h at 37°C and then analyzed for caspase-3 catalytic activity using the fluorogenic assay (Nicholson et al., 1995; Thornberry et al., 1997a). The data have been corrected to exclude fluorescence activity resulting from direct cleavage of the substrate by the activator caspases themselves. The inset shows a titration of Hsp60 on caspase-6-mediated activation of caspase-3 cleavage activity. (B) Immunoblot analysis (R280 antibody) of caspase-3 after incubation with Hsp60 alone (top panel) or Hsp60 pre-treated with ATPγS (bottom panel) and then followed by treatment with the activator caspase as described in (A). Lanes 2–4 in both panels contain activator caspases. (C) The effect of ATPγS on Hsp60-mediated activation of caspase-3 following the initial pre-incubation of pro-caspase-3 with Hsp60. ATPγS was added to the reaction mixture at the onset (pre-incubation) or following (post-incubation) the initial 10 min room temperature incubation between Hsp60 and pro-caspase-3. Caspase-6 was added to the reactions immediately following the initial pre-incubation step and the samples were incubated further at 37°C for 2 h. Following incubation, the samples were then analyzed for caspase-3 catalytic activity by fluorometry (Nicholson et al., 1995; Thornberry et al., 1997a). The data in (A) and (C) represent the average of three experiments.

Discussion

Multicellular organisms have evolved a number of molecular mechanisms for responding to cellular damage and environmental stress. Included among these is the induction of a subfamily of heat shock proteins designated as chaperonins (Hsp60, Hsp70 and Hsp90). Known functions of the chaperonins include folding, assembly and translocation of other proteins (Hartl and Martin, 1995; Neupert, 1997; Beissinger and Buchner, 1998; Bukau and Horwich, 1998). Taken together, the experiments described herein suggest a novel role for Hsp60 in facilitating the activation of caspase-3 by upstream group III caspases.
caspases-3 and -6 as well as other as yet unidentified proteins. Among the group III activator caspases screened, that potentially could catalyze maturation of caspase-3, only caspase-6 was detected. This finding is consistent with data from a previous study showing that activated caspase-6 could catalyze cleavage and activation of caspase-3 in extracts incubated at 37°C (Liu et al., 1996a; Orth et al., 1996). Interestingly, caspase-9 was not identified as part of the affinity-purified activation complex. However, the absence of detectable caspase-9 does not imply that the caspase-9–Apaf-1 pathway serves no role in camptothecin-induced apoptosis. One possibility is that caspase-6-mediated activation of pro-caspase-3 is part of an amplification loop which also involves caspase-9, at least initially. For example, in response to an apoptotic stimulus, caspase-9 would activate caspase-3, which in turn could activate caspase-6. One or both of these steps may be Hsp assisted. Furthermore, it has been demonstrated previously that caspase-6 is a substrate of caspase-3 (Hirata et al., 1998; Kawahara et al., 1998). Therefore, caspase-3 could be shunted into an amplification loop through reciprocal activation with caspase-6. In essence, the contribution of caspase-9 to this process, while significant for initiation of the amplification loop, would be relatively minor.

Characterization of Hsp60 function in the context of a purified in vitro system revealed that Hsp60 facilitates the activation of pro-caspase-3 by different upstream caspases, including caspase-6. Several lines of evidence support the notion that the interaction between caspases and Hsp60 is physiologically relevant. First, Hsp60 co-purified with caspase-3 through the chromatography procedure, despite stringent washing conditions during affinity purification. Secondly, Hsp60 co-immunoprecipitated with caspase-3 from extracts of metabolically labeled cells. Thirdly, Hsp60-assisted activation of pro-caspase-3 was dependent on Hsp60 catalytic function as indicated by the requirement for ATP hydrolysis. Fourthly, it has been demonstrated that both caspase-3 and Hsp60 localize to the mitochondrion (Mancini et al., 1998), although the possibility that the interaction also occurs in an extra-mitochondrial environment cannot be excluded. In the accompanying manuscript, Samali et al. (1999) report findings similar to our own. Using an in vitro model of cytochrome c/dATP-mediated apoptosis, they show that Hsp60 in combination with Hsp10 accelerates maturation of caspase-3. They also describe concomitant mitochondrial activation of caspase-3 and release of cytochrome c and Hsp60/10. Consistent with our study, they propose that Hsp60 participates in caspase-3 activation by acting as a chaperone to promote maintenance of pro-caspase-3 in a protease-sensitive state.

While in many cases, an anti-apoptotic function has been ascribed to different chaperonins (Mosser et al., 1995, 1996). Consistent with the caspase–Hsp60 interaction described herein, Hsp27 has been shown to co-precipitate with granzyme B in cytoplasmic lysates and is believed to participate in CTL-mediated apoptosis (Berendsen et al., 1998). Similarly, the anti-apoptotic Bcl-2-interacting BAG-1 protein was found to associate with 70 kDa heat shock proteins (Hsp70/Hsc70) and is thought to regulate their activity by serving as a nucleotide exchange factor (Hohfeld, 1998; Stuart et al., 1998).

Together, these studies reveal an important link between chaperonins and proteins known to modulate apoptosis. Since apoptosis is considered a normal and necessary event in many physiological paradigms (e.g. development, cell turnover, cell injury), it follows that one important aspect of the heat shock stress response would be to modulate the balance between cell death and survival.

Materials and methods

Cloning and preparation of recombinant caspase proteins

Recombinant pro-caspase-3 containing a C-terminal His6 tag was generated by PCR amplification and confirmed by DNA sequence analysis. DNA sequences encoding the fusion protein were cloned into an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible bacterial expression vector (pET11D, Novagen) and transformed into BL21 cells (Novagen). A 2 l culture of cells derived from a single BL21 transformant was grown overnight at 30°C to a final optical density (OD600) of 0.6. Expression of the pro-caspase-3 fusion protein was induced by treating the culture with 1 mM IPTG for 2 h at room temperature. The culture was harvested by centrifugation (10 000 g) and the pellet was lysed, on ice, by sonication in a buffer containing 20 mM imidazole, 0.5 M NaCl, 5 mM β-mercaptoethanol, 0.1% Triton X-100, 20 mM Tris–HCl pH 8.0. The lysates were clarified by centrifugation (10 000 g) and purified by nickel-chelate FPLC (Ni-NTA-superflow, Qiagen) chromatography at 4°C. Following loading of the lysate and an initial wash of the column with lysis buffer (without Triton X-100), fractions were eluted from the column using a 20–250 mM imidazole gradient in lysis buffer (without Triton X-100) and then supplemented with 10 mM dithiothreitol (DTT) and 1 mM EDTA. Column fractions were analyzed by SDS–PAGE on 4–20% gels and protein concentrations were determined using the Bradford assay (Bio-Rad). Recombinant caspases-6, -8 and -9 proteins were expressed and purified as described previously (Nicholson et al., 1995; Thornberry et al., 1997a).

Preparation of extracts and purification of IETD-binding proteins (CAPS)

Whole-cell extracts were prepared from 32 l of Jurkat cells (1×10^6 cells/l) grown in RPMI/10% fetal calf serum and treated for 4 h with camptothecin (10 μM) or anti-Fas antibody (0.1 μg/ml). Cultures were harvested by centrifugation (1000 g) and cell pellets were lysed in ICE buffer III (50 mM HEPES-KOH pH 7.0, 0.1% CHAPS, 2 mM EDTA, 10 mM DTT, 10% glycerol) by Dounce homogenization. The lysates were clarified through successive centrifugation steps (1000 g spin followed by a 10 000 g spin) at 4°C and the crude extracts were frozen on dry ice and stored at –80°C until further use.

Protein purification was performed at 4°C and, whenever possible, samples were kept on ice. All chromatographic steps were performed on a Pharmacia FPLC system. Crude Jurkat cell extract (450 mg of total protein) was loaded onto a Hi-load 16/100 SuperNovagel (Pharmacia) and washed with five column volumes of ICE buffer III. The bound protein was eluted with a linear gradient of 0–0.8 M NaCl in ICE buffer III. In this and all subsequent chromatographic steps, protein concentration was monitored by continuous UV absorption at 280 nm. The active fractions from the Hi-load Q-Sepharose column (as determined by cleavage of a fluorogenic substrate) were pooled, diluted 3-fold in ICE buffer III and incubated in the presence of 0.5 μM biotin-IETD-CHO at room temperature for 30 min. The inhibitor–protein complexes were captured on a 1.0 ml streptavidin–agarose affinity column equilibrated in ICE buffer III. To dissociate proteins that bound non-specifically to the resin, the biotin-IETD-CHO affinity column was washed with ICE buffer III containing 0.5 M NaCl and then re-equilibrated to 50 mM NaCl in the same buffer. Fractions were eluted...
from the affinity column in two stages. The first elution was carried out immediately after the initial wash steps, and in the presence of 10 μM IETD-CHO. The second elution was performed following an overnight incubation of the resin in IETD-CHO-containing buffer. Column fractions were resolved on 4–20% SDS–polyacrylamide gels and proteins were visualized by silver staining.

**Peptide sequence analysis**

Pooled fractions of purified protein from the IETD-CHO affinity fractionation were precipitated with 20% trichloroacetic acid and resolved on a 4–20% SDS–polyacrylamide gel. Ten percent of the material loaded onto the gel was resolved in a parallel lane that was silver stained and used to localize the position of individual polypeptides in the untreated portion of the gel. Once the position of each polypeptide was established, the corresponding gel slice was excised, rinsed in water and immersed in a solution of 50% acetonitrile:water. A gel slice containing two polypeptides was eluted at a wt. of ~60 kDa was characterized further by tandem mass spectrometry (MSMS) peptide sequence analysis (Harvard Microchem.). The primary sequence of two confirmed peptides is as follows: MSpep1, TVIEQSWGSPK; and Mspep2, ISSIQSIVPALEIAN-AHR. A search of the DDBJ/EMBL/GenBank database showed that both sequences corresponded to human Hsp60 (accession No. A32800).

**Immunoblot analysis**

Immunoblot analysis was carried out as described previously (Rasper et al., 1998). With the exception of the caspase-6 antisemur, polyclonal antisera for caspases 1–9 were raised in rabbits against each of the respective human recombinant caspase large subunits (p20). The caspase-6 antisemur was raised against the small subunit (p10) of caspase-6. The Apaf-1 antisemur was raised against the recombinant full-length human Apaf-1 as described (Zou et al., 1997). The human Hsp60 antibody (goat anti-human polyclonal) was purchased commercially (Stressgen) and used as per the manufacturer’s recommendations.

**Immunoprecipitations**

HeLa cells were metabolically labeled with 100 μCi/ml of Trans35S-label for 60 min, prior to lysis as described (Casciola-Rosen et al., 1996). Cell lysates were immunoprecipitated with normal rabbit serum, polyclonal rabbit serum to the caspase-3 precursor (MF393) (Mancini et al., 1998) or goat polyclonal anti-Hsp60 (Stressgen), and subjected to SDS–PAGE and autoradiography. Co-immunoprecipitation of Hsp60 with caspase-3 and caspase-6 from the ion-exchange chromatography fractions was performed as follows. A 220 μl aliquot of the active Hi-load Q fractions (see above) was incubated for 1 h at 4°C with 800 μl of ICE buffer III and 2 μl of either an anti-caspase-3 (MF393) or an anti-caspase-6 (MF424) polyclonal antibody. Following incubation, 100 μl of a 50% bovine serum albumin (BSA)-blocked protein A–Sepharose slurry was added to each and the beads were then washed four times with 500 μl of ICE buffer III. After removal of the last wash buffer, proteins were eluted from the beads with 80 μl of Laemmli SDS-containing sample buffer, followed by denaturation for 5 min at 95°C. The resulting supernatants were resolved on SDS–polyacrylamide gels and transferred to nitrocellulose by electroblotting. The blots were then probed with an anti-Hsp60 antibody (Stressgen) and visualized using enhanced chemiluminescence (ECL) detection (Rasper et al., 1998).

**Caspase activity assays**

Fluorogenic assays were performed using the DEVD-amino-methyl-coumarin (AMC) and/or IETD-AMC tetrapeptide substrates as described previously (Thornberry et al., 1997a). For the caspase-3 activation experiments, affinity-purified pro-caspase-3 (0.04 μM) was incubated alone or in different combinations (as defined in the figure legends) with 1.0 mM ATP-MgCl2 or 1.0 mM ATP alone or in different combinations (as defined in the figure legends) with 1.0 mM ATP-MgCl2 or 1.0 mM ATP. The reactions were then diluted to 200 μl with ICE buffer III in the presence of the DEVD-AMC substrate (10 μM), and cleavage activity was monitored at room temperature by fluorometry (Nicholson et al., 1995; Thornberry et al., 1997a). PARP cleavage assays were performed using a [135S]methionine-radioabeled PARP substrate as previously described (Casciola-Rosen et al., 1996).

**Acknowledgements**

We would like to thank Nancy Thornberry for kindly providing us with purified recombinant activator caspases. We would also like to thank Stefan Nobel and Francois Gervais for their critical reading of the manuscript and for many helpful discussions.

**References**


