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Caspases: their intracellular localization and translocation during apoptosis

Boris Zhivotovsky[1], Afshin Samali[1], Annie Gahm[1] and Sten Orrenius*[,1]

[1] Division of Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Box 210, S-171 77 Stockholm, Sweden

* corresponding author: Division of Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Box 210, S-171 77 Stockholm, Sweden. tel: +46 8 728 75 90 Fax: +46 8 32 90 41

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Abstract

The activation of the caspase family of proteases has been detected in numerous cell systems and appears to function as a common pathway through which apoptotic mechanisms may operate. Caspases are synthesized as precursors (pro-caspases) and are converted into mature enzymes by apoptotic signals. The effects of caspases in apoptosis are accomplished by the cleavage of numerous proteins located in different intracellular compartments. In the present study we have addressed the question of the subcellular localization of different pro- and active caspases as well as several other proteins, such as Apaf-1, calpain and DFF, which also play important roles in the apoptotic process. We found that at least three pro-caspases (pro-caspases-2, -3 and -9) were present in both the mitochondrial and cytosolic fractions of untreated Jurkat T lymphocytes. Only pro-caspase-2 was found in the nuclear fraction. Pro-caspases-7 and -8 were found only in the cytosolic fraction. In apoptotic cells, caspases-3, -8 and -9 were present in the cytosolic fraction, whereas caspases-3 and -9 were also found in the mitochondrial fraction and caspase-7 in the microsomal fraction. Caspases-2 and -3 were present in the nuclear fraction. The selective localization of pro-caspases in different subcellular compartments may play an important, but yet unknown, role in their activation. The translocation of active caspases to other subcellular compartments appears to be critical for the development of the apoptotic process.

Keywords: apoptosis; caspases; intracellular localization; activation

Abbreviations: AK, adenylate kinase; Apaf-1, apoptotic protease activating factor; DFF, DNA fragmentation factor; ER, endoplasmic reticulum; ICAD, inhibitor of caspase-activated DNase; PARP, poly(ADP-ribose)polymerase

Introduction

The cellular proteolytic machinery includes numerous proteases localized in different intracellular compartments. This machinery functions to maintain fundamental cellular processes and to remove denatured or misfolded proteins. The precise intracellular localization and function of many of the proteases are unknown. The requirement for proteolysis during apoptosis is well documented (for review see[1,2]). During the last few years the 'main players' in the induction and execution steps of the apoptotic process were identified as a family of aspartic acid-specific cysteine proteases, the caspases.[3] At the present time, fourteen members of this family of proteases have been characterized and their genes have been cloned, although not all of these members are human proteins.[2]

Caspases are synthesized as zymogens, and an apoptotic signal converts the precursors into mature enzymes. Caspases differ in the length and sequence of their N-terminal prodomain and can be divided into two subgroups; pro-caspases-1, -2, -4, -5, -8, -9, -10 and -14 with long prodomains and pro-caspases-3, -6, -7, -11 and -13 with short prodomains. In the long prodomain-containing group of caspases two distinct modules essential for protein-protein interaction have been identified. The first is referred to as the death effector domain (DED) and is present in both pro-caspases-8 and -10.[4,5] The second is termed caspase recruitment domain (CARD) and is found in pro-caspases-1, -2, -4 and -9. The CARD domain is also present in Apaf-1, the mammalian homolog of the CED-4 protein.[6,7] It seems that most, if not all, of the long prodomain-containing caspases are activated via oligomerization-induced autoproteolysis.[8,9] For example, the oligomerization of pro-caspases-2 and -8 at the plasma membrane is sufficient for their autoactivation.[10,11] The activation of pro-caspase-9 requires binding to Apaf-1 and the presence of at least two other components, i.e. dATP and cytochrome *c*.[12] Apaf-1 first forms oligomers and then facilitates pro-caspase-9 autoactivation by oligomerizing its precursor molecules.[13] On the other hand, the activation of short prodomain-containing caspases requires an initial first step cleavage, e.g. by active caspases or granzyme B, followed by a second autoproteolytic step. For full activation short prodomain-contained caspases requires also cleavage in the interdomain linker.[14] Thus, activated caspase-9 can initiate a caspase cascade involving the downstream executioners, caspases-3, -6 and -7.

All members of the caspase family share a number of amino acid residues crucial for substrate binding and catalysis. Despite their uniform requirement for an aspartate residue in the P_1 position of the substrate, caspases can be divided into three different groups

according to their substrate preferences.[15] The predicted caspase specificities correspond to the cleavage sites in the different intracellular target proteins. The effects of caspases in apoptosis appear to be accomplished by the cleavage of a large number of proteins located in the cell membranes, cytoplasm and nucleus, although the significance of the particular cleavages for the cell death process is still unclear (for review see[1,16]).

It has been suggested[17] that pro-caspases serve as the 'cytoplasmic regulator' of apoptosis which, after being activated, can translocate to other intracellular compartments, such as the endoplasmic reticulum (ER) and the nucleus. However, it has recently been demonstrated that, although the majority of pro-caspase-3 is present in the cytosol, a sizeable fraction of this protein has a mitochondrial location.[18,19] The presence of pro-caspase-3 in the mitochondrial fraction was found not to be restricted to a specific cell type but was observed in different cells and tissues. The presence of pro-caspase-2 in the nuclear fraction has also been demonstrated recently.[20] The exact intracellular localization of many other pro-caspases and caspases is still unclear. In the present study we have therefore addressed the question of the subcellular localization of different pro- and active caspases and of some other proteins, i.e. Apaf-1, calpain and DFF, which play important roles in the apoptotic process. We report that several of the known pro-caspases are found in multiple intracellular compartments and translocate from these compartments to others upon activation. During the preparation of this manuscript a similar finding concerning the mitochondrial localization of pro-caspase-2 and -9 was reported.[21] Thus, the intracellular localization of pro-caspases and translocation of activated caspases appears to be critical for the development of the apoptotic process.

Results

Characterization of subcellular fractions

Four subcellular fractions were isolated from Jurkat cells, and the purity of these fractions was tested using specific marker enzymes (Table 1). The mitochondrial fraction contained minor contamination by microsomes (5–8%) and cytosol (2%), which were also present in trace amounts in the nuclear fraction. The microsomal fraction contained relatively high cytochrome *c* oxidase activity (12.2%), which reflected the co-sedimentation of light mitochondria with microsomes. Western blot analysis of marker proteins revealed the presence of lamin B and PARP only in the nuclear fraction, AK-1 in the cytosol and AK-2 in the mitochondrial fraction (Figure 1). Activities of marker enzymes for lysosomes and Golgi vesicles, acid phosphatase and UDP-galactosyltransferase, respectively, were not detectable in any of the fractions. Thus, the isolated subfractions were considered to be of sufficient purity for the purpose of our study.

Subcellular localization of pro-caspases

The intracellular localization of different pro-caspases in Jurkat cells was determined by Western blotting. Pro-caspase-1 was not found in any of the fractions tested with any of the anti-caspase-1 Abs used. Although the majority of native 51 kDa pro-caspase-2 protein was found in the cytosolic fraction, significant amounts of this protein were detected also in the nuclear and mitochondrial fractions (Figure 2). Western blot analysis of pro-caspase-3 in different subcellular fractions revealed the presence of the 32 kDa pro-enzyme in the soluble cytosol and mitochondria (Figure 3). The presence of a small amount of pro-caspase-3 in the microsomal fraction can be explained by contamination of this fraction with light mitochondria (c.f. Table 1). Pro-caspase-7, molecular weight 35 kDa, was detected only in the cytosolic fraction of Jurkat cells (Figure 2). The full-length pro-caspase-8, which exists in two isoforms, pro-caspase-8/a and pro-caspase-8/b, with molecular weight 55–53 kDa,[22] was also detected only in the cytosolic fraction (Figure 2). In contrast, pro-caspase-9, molecular weight 46 kDa, was observed in two subcellular fractions. The majority of this pro-caspase was present in the cytosolic fraction but a small amount was also found in the mitochondria (Figure 2). Thus, at least three pro-caspases (pro-caspases-2, -3 and -9) are found in both the cytosolic and the mitochondrial fractions, whereas pro-caspase-2 is also present in the nuclear fraction.

Table 1 Marker enzyme activities in subcellular fractions obtained from untreated or apoptotic Jurkat cells

Fraction[a]	Protein (%)	LDH (%)	Cyt. *c* ox (%)	NADH-cyt. *c*. reductase	NADPH-cyt. *c* reductase	IDPase
Untreated cells						
Cytosol	49.1	91.8	1.1	2.5	3.1	5.2
Mitochondria	26.2	2.3	83.6	4.8	7.8	6.8
Nuclei	11.2	2.7	3.3	3.9	2.6	4.0
Microsomes	13.5	3.2	12.0	88.8	86.5	84.0
Apoptotic cells						
Cytosol	50.2	90.9	1.2	2.9	3.4	5.5
Mitochondria	26.8	2.4	83.7	5.0	7.6	7.1
Nuclei	10.9	2.5	3.6	3.6	2.9	3.7
Microsomes	12.1	4.2	11.5	88.5	86.1	83.7

[a]The amount in each fraction is expressed as a percentage of the sum of the amounts recovered in all fractions. The data presented are from one experiment typical of five

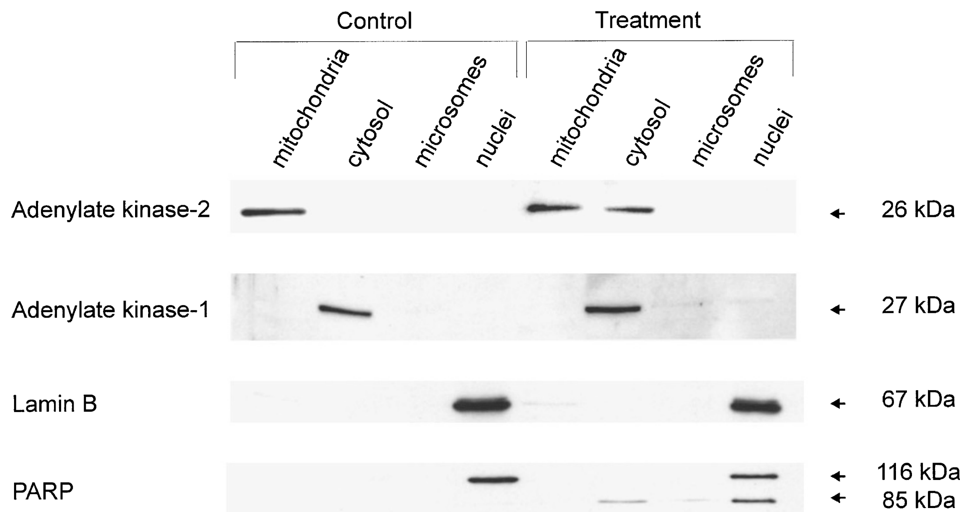

Figure 1 Subcellular localization of marker proteins in Jurkat cells. Cytosol, mitochondria, microsomes and nuclei were isolated from untreated cells or cells treated with anti-CD95 Abs as described in Materials and Methods (similar distribution was observed when cells were treated with etoposide, although the time frame for this re-distribution was different). Protein samples were prepared for Western blotting and membranes were probed with antiserum against AK1, AK2, Lamin B and PARP, respectively

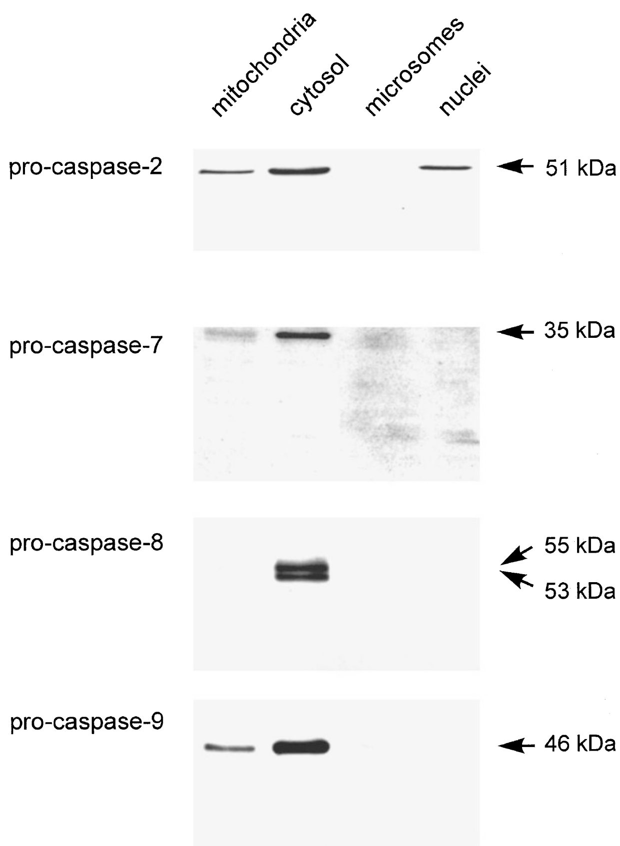

Figure 2 Subcellular distribution of pro-caspases in Jurkat cells. Cytosol, mitochondria, microsomes and nuclei were isolated from untreated Jurkat cells as described in Materials and Methods. Protein samples were prepared for Western blotting and membranes were probed chronologically with antiserum against pro-caspases-2, -7, -8 and -9

Subcellular localization of apoptosis-related proteins

It is well known that several proteins, such as Apaf-1, DFF-45 and calpain, also play an important role in the apoptotic process. Apaf-1, is a component of the 'apoptosome' complex and involved in the activation of pro-caspase-9 in the presence of cytochrome *c* and dATP.[12] DFF is a heterodimer consisting of two subunits, DFF-45/ICAD and DFF-40/CAD.[23,24] DFF-45 is cleaved by caspase-3. Cleavage of DFF-45 frees DFF-40 from the complex and thus initiates DNA fragmentation during apoptosis. The activity of μ-calpain has been implicated in several experimental models of apoptosis, although it is unclear whether this activity is located up- or downstream of caspase activation.[25,26] To investigate the intracellular localization of these proteins, extracts from the four subcellular fractions described above were analyzed for the presence of Apaf-1, DFF-45 and μ-calpain by Western blotting. Apaf-1, a 130 kDa protein, as well as μ-calpain, a 80 kDa protein, were detected only in the cytosolic fraction, whereas DFF-45 was also found in the nuclear fraction of untreated cells (Figure 4).

Subcellular re-distribution of active caspases and apoptosis-related proteins

To investigate if activated caspases and apoptosis-related proteins were present in the same subcellular fractions as their precursors, or were translocated into other intracellular compartments, protein extracts isolated from the cytosolic, mitochondrial, microsomal and nuclear fractions of apoptotic cells were subjected to Western blotting. The purity of these fractions was also tested, using the same enzyme markers as described above, and found to be comparable to the purity of the corresponding fractions from untreated cells (Table 1). As shown in Figure 1, AK-1 was still recovered in the cytosolic

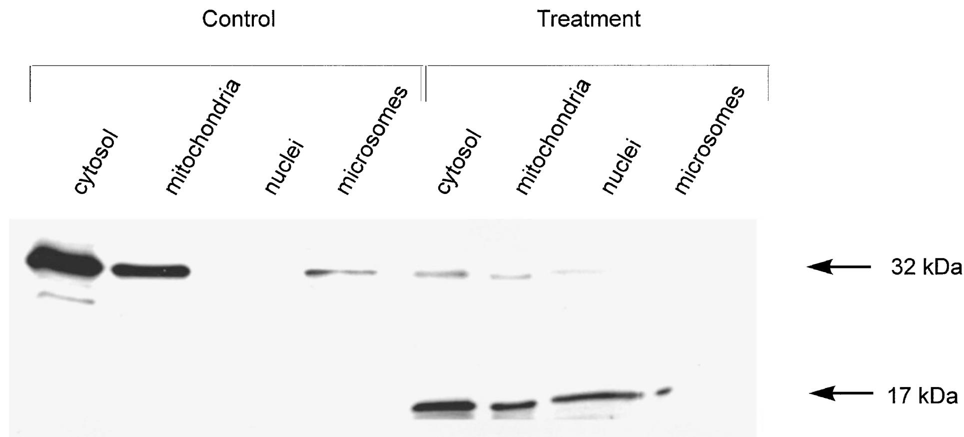

Figure 3 Subcellular localization of pro- and active caspase-3 in Jurkat cells. Cytosol, mitochondria, microsomes and nuclei were isolated from untreated cells or cells treated with etoposide as described in Materials and Methods (similar distribution was observed when cells were treated with anti-CD95 Abs, although the time frame for this re-distribution was different). Protein samples were prepared for Western blotting and membranes were probed with antiserum against p17 fragment of caspase-3

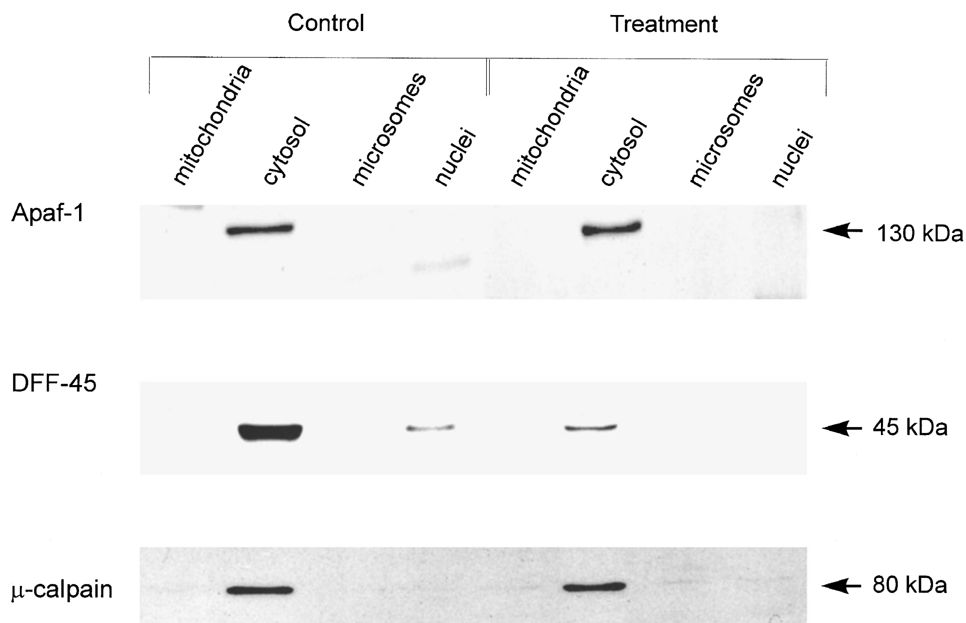

Figure 4 Subcellular localization of apoptosis-related proteins in Jurkat cells. Cytosol, mitochondria, microsomes and nuclei were isolated from untreated cells or cells treated with etoposide as described in Materials and Methods (similar distribution was observed when cells were treated with anti-CD95 Abs, although the time frame for this re-distribution was different). Protein samples were prepared for Western blotting and membranes were probed with antiserum against Apaf-1, DFF-45 and μ-calpain, respectively

fraction in apoptotic cells, whereas a significant amount of AK-2, which is localized in the intermembrane space of mitochondria of untreated cells, was found in the cytosolic fraction from apoptotic cells (Figure 1). Recently we have shown that the release of AK-2 from the mitochondrial intermembrane space into the cytosol is a consequence of apoptosis, rather than a leakage of mitochondria.[27] Both lamin B and PARP were present in the nuclear fraction of apoptotic cells (Figure 1). In contrast to lamin B proteolysis, the cleavage of PARP is a relatively early event in apoptosis. In Jurkat cells undergoing apoptosis the cleavage of PARP preceded that of lamin B by several hours (Zhivotovsky, B., unpublished observation). In our experiments, subcellular fractions were isolated 2 h after treatment of Jurkat cells with anti-CD95 Abs and 4 h after treatment with etoposide, i.e. long before any apparent damage to the nuclear and mitochondrial membranes could be observed (data not shown). At these time points no lamin B cleavage products were observed, while PARP cleavage was detected (Figure 1). Both Apaf-1 and μ-calpain were detected in the cytosolic fraction of apoptotic cells (Figure 4). Moreover, the amount of the native form of these proteins was similar to that in control

cells. The level of DFF-45 was much lower in the cytosol from apoptotic cells and was undetectable in the nuclear fraction of apoptotic cells (Figure 4). The decrease in the level of DFF-45 in apoptotic cells could be explained by its cleavage in these intracellular compartments. However, the absence of the cleavage products in the blots is because of the specificity of antibodies, which can not recognize the fragments of DFF-45.

Analysis of the subcellular localization of active caspases in apoptotic cells revealed a pattern different from that seen in untreated cells. Thus, while pro-caspase-2 was localized in the cytosol, mitochondria and nuclei of untreated cells, active caspase-2, which consists of two cleaved fragments (p32 and p18) was found only in the nuclear fraction (Figure 5). The p17 fragment of active caspase-3 was detected in the cytosolic, mitochondrial and nuclear fractions of treated Jurkat cells (Figure 3). Redistribution of pro- and active forms of caspase-7 from the cytosolic to the microsomal fraction was observed in apoptotic cells (Figure 3). The cleavage product of pro-caspase-8, the p43/p41 doublet, was detectable in the cytosol, whereas a certain amount of pro-caspase-8 was also associated with the mitochondrial, microsomal and nuclear fractions. The active fragment of pro-caspase-9, on the other hand, was found primarily in the cytosol, but a small amount was also detected in the mitochondrial fraction (Figure 5).

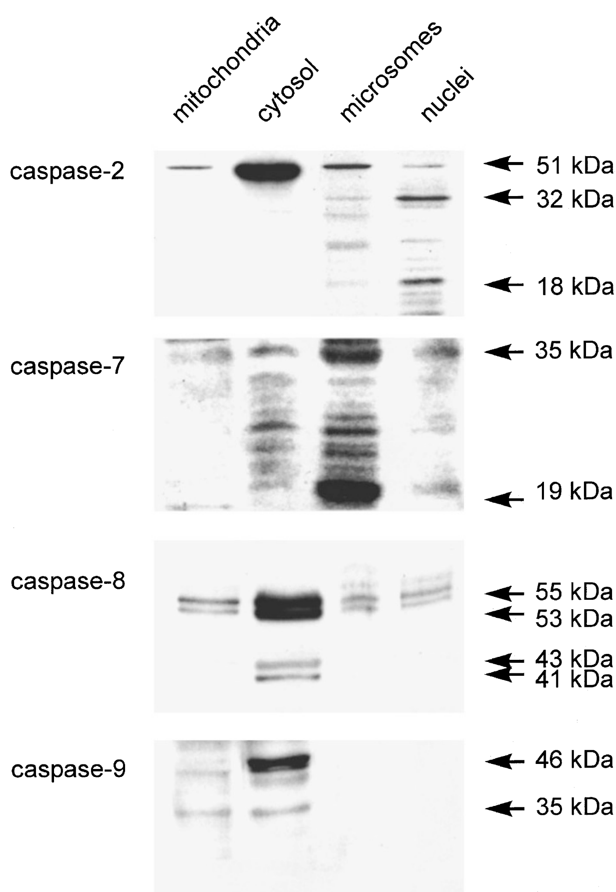

Figure 5 Subcellular localization of active caspases in apoptotic Jurkat cells. Cytosol, mitochondria, microsomes and nuclei were isolated from cells treated with etoposide as described in Materials and Methods (similar distribution was observed when cells were treated with anti-CD95 Abs, although the time frame for this re-distribution was different). Protein samples were prepared for Western blotting and membranes were probed with antiserum against caspases-2, -7, -8 and -9, respectively

Discussion

In apoptotic cells the cleavage of more than seventy proteins by caspases has been observed (for review see[1,16]). Since these proteins are located in different intracellular compartments, it is reasonable to assume that the caspases are present in the same compartments, or that they translocate to these compartments upon activation. The current study was undertaken to test this hypothesis. We found that at least three pro-caspases (-2, -3 and -9) were present in both the mitochondrial and cytosolic fractions of untreated Jurkat cells. Amongst these, pro-caspase-2 was also present in the nuclear fraction. As was mentioned above, a similar finding concerning the mitochondrial localization of pro-caspases-2 and -9 was reported recently by Kroemer and co-workers.[21] Pro-caspases-7 and -8 were found only in cytosolic fraction. In apoptotic cells caspases-3, -8 and -9 were present in the cytosolic fraction, whereas caspases-3 and -9 were also found in the mitochondrial fraction and caspase-7 in the microsomal fraction. Caspases-2 and-3 were present in the nuclear fraction. In accordance with a previous report[28] we did not detect pro-caspase-1 in our Jurkat cells. However, the involvement of caspase-1 in apoptosis may differ between cell types. For example, it was recently reported that in TNF-treated HeLa cells, pro-caspase-1 was translocated to the nucleus, where it was activated.[29] On the other hand, the only known substrates for caspase-1 are the cytokines, pro-interleukin-1β and pro-interleukin-18,[2,30] as well as phospholipase A2,[31] and no nuclear targets have been identified. Thus, as yet it is unclear what contribution nuclear caspase-1 would make to the proteolytic cascade in apoptosis.

Although pro-caspase-2 was found in cytosol, mitochondria and nuclei of untreated cells, activated caspase-2 was localized only in the nuclear fraction of apoptotic Jurkat cells. Using fluorescent protein fusion constructs, the localization of both precursor and processed caspase-2 in the cytoplasmic and the nuclear compartments has recently been shown.[20] It is also possible that activation of pro-caspase-2 in cytosol occurs later than in the nuclei, given that activated caspase-2 has been previously detected in several types of apoptotic cells.[32,33,34] Despite evidence for the involvement of caspase-2 in apoptosis, no protein targets for this caspase have yet been found.

Cytoplasmic pro-caspase-3 plays a central role in the execution step of apoptosis, where it controls both cytoplasmic and nuclear events associated with the process.[2,28,35] Although two groups recently reported that pro-caspase-3 also has a mitochondrial localization,[18,19] the function of pro-caspase-3 in mitochondria, as well as the subcellular distribution of caspase-3 in apoptotic cells, were unclear. The present study has shown that active p17 polypeptide of caspase-3 was present in the cytosolic, mitochondrial and nuclear fractions of Jurkat cell undergoing apoptosis. This distribution is in agreement with the

known localization of many substrates for caspase-3 (for reviews see[1,16]). One of the potential targets for caspase-3 in the mitochondria is Bcl-2. Indeed, Bcl-2 was shown to be cleaved *in vitro* by recombinant caspase-3.[36] Furthermore, we have recently shown that chemotherapy-induced apoptosis in myeloid leukemic cells was associated with the cleavage of endogenous Bcl-2, and that this event preceded the proteolysis of both the nuclear caspase-3 substrate, PARP, and of the caspase-6 substrate, lamin B.[37]

The intracellular distribution of pro- and active caspase-7 reported here is in agreement with the recent finding that caspase-7 was exclusively associated with the endoplasmic reticulum of mouse hepatocytes, where it cleaved the ER-specific substrate, sterol regulatory element-binding protein-1 (SREBP).[38]

Pro-caspase-8 was found exclusively in the cytosolic fraction of untreated cells. In apoptotic cells, this proenzyme was present also in other intracellular compartments, such as the ER and the nucleus. It has been reported that pro-caspase-8 is present in the ER in a complex with Bcl-2/Bcl-X_L and p28 Bap31 proteins,[39] and that this complex might cooperate with the mitochondria 'to control proximal and distal steps in a Bcl-2-regulated caspase cascade'. Since Bcl-2 is also found in the nuclear membrane, it is possible that a similar complex may be associated with the nuclear membrane as well. The presence of caspase-8 in the cytosol is consistent with its proposed role in the cleavage of the cytoplasmic protein Bid with subsequent activation of the cytochrome *c* release from mitochondria[40,41] and in the activation of cytosolic pro-caspase-3 without the requirement for an accelerator, such as caspase-9.[14]

Caspase-9 was detected in the soluble cytoplasm and, in trace amounts, also in the mitochondria of apoptotic cells. In the cytoplasm pro-caspase-9 binds to Apaf-1 and is activated to caspase-9 in the presence of cytochrome *c* and dATP. Active caspase-9 subsequently activates pro-caspase-3.[12] Whether a similar activation mechanism exists also for mitochondrial pro-caspase-9 is still unclear. We were not able to detect Apaf-1 in the mitochondrial fraction from Jurkat cells, suggesting that, if it was at all present, the amount of Apaf-1 was below the level of detection with the Abs used in our study. It has been suggested that, unlike the other caspases, caspase-9 has an unusually active zymogen and that proteolysis of procaspase-9 does not seem to be required for activation.[42] Indeed, processing in the interdomain linker, characteristic for many caspases, enhances caspase-9.[42] It could be that the mitochondrial pro-caspase-9 might undergo activation within the mitochondria without the formation of an apoptosome complex. It has been shown that the long prodomain pro-caspases are able to autoactivate via oligomerization, although this activation requires high zymogen concentration.[8,9] While this manuscript was being reviewed, three groups identified novel CARD-containing protein with homology to herpesvirus-2 protein E10 that promotes the activation of pro-caspase-9.[43–45] Although the CARD of mammalian E10 does not directly interact with caspase-9, its C-terminal region required for the activation of pro-caspase-9, suggesting a mode of pro-caspase-9 activation that is independent of Apaf-1.[45] If mammalian homologue of viral E10 or some unknown proteins with a similar structure have mitochondrial location, theoretically it will be possible that pro-caspase-9 may be activated within the mitochondria, perhaps with subsequent activation of pro-caspase-3. Under this scenario, mitochondrial Bcl-2 could be cleaved leading to the release of mitochondrial constituents (cytochrome *c*, AK-2, etc.) into the cytoplasm and amplification of the caspase cascade by the apoptosome mechanism. However, further work is required to determine if, and how, the activation of the mitochondrial pool of pro-caspases contributes to the proteolytic cascade in apoptosis.

Materials and Methods

Antibodies and chemicals

Anti-caspase-1 Abs were from Calbiochem (Cambridge, MA, USA), anti-caspase-2 Abs were from Transduction Laboratories (Lexington, KY, USA), anti-caspase-3 Abs were provided by Dr. DW Nicholson (Merck Frosst Center for Therapeutic Research, Quebec, Canada), anti-caspase-7 Abs were raised in our laboratory against the peptide CKPDRRSSFVPSLFSKK from the p20 subunit of caspase-7,[46] anti-caspase-8 Abs were donated by Dr. PH Krammer (German Center for Cancer Research, Heidelberg, Germany), anti-caspase-9, anti-Apaf-1 and anti-DFF-45 Abs were donated by Dr. X Wang (University of Texas Southwestern Medical Center at Dallas, TX, USA), anti-AK-1 and -AK-2 Abs were from Dr. T Noma (Yamaguchi University School of Medicine, Yamaguchi, Japan), anti-lamin Abs were from Novocastra Laboratories, Newcastle upon Tyne, UK, anti-PARP Abs were from Biomol (Plymouth Meeting, PA, USA), anti-μ-calpain Abs were from Chemicon International Inc. (Temecula, CA, USA), goat anti-rabbit and goat anti-mouse IgG-HRP were from Pierce (Rockford, IL, USA). ECL was from Amersham Corp. (Buckinghamshire, UK). All protease inhibitors were from Roche Diagnostics Scandinavia AB (Bromma, Sweden). All other chemicals were from Sigma (St. Louis, MO, USA).

Cell culture and induction of apoptosis

Jurkat cells (human leukemia T cell line, obtained from the European Collection of Cell Cultures, Salisbury, UK) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C. Cells were maintained in a logarithmic growth phase by routine passage every 3–4 days. Apoptosis was induced by treatment with 6 μg/ml etoposide (Vepesid, VP-16, Bristol-Myers, Solna, Sweden), or 250 ng/ml anti-CD95 mAb (clone CH-11, Medical & Biological Laboratories, Ltd., Nagoya, Japan).

Preparation of different subcellular fractions from Jurkat cells

Jurkat cells were untreated, etoposide-treated, or stimulated with anti-CD95 Abs. They were washed once with buffer A (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4) and then resuspended in buffer B (buffer A plus 5% Percoll, 0.01% digitonin, and a cocktail of protease

inhibitors containing: 10 μM aprotinin, 10 μM pepstatin A, 10 μM leupeptin and 1 mM PMSF). After incubation on ice for 15 min, the suspension was centrifuged to remove unbroken cells and nuclei ($2500 \times g$, 10 min). The mitochondria were then pelleted by centrifugation at $15\,000 \times g$ for 15 min and resuspended in buffer C (300 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4, supplemented with the cocktail of protease inhibitors). The supernatant was further centrifuged at $100\,000 \times g$ for 1 h. The resultant supernatant was designated as cytosol and the pellet as the microsomal fraction. The nuclear pellet was resuspended in 10 mM Tris-HCl, pH 7.5, 2.5 mM KCl, 2.5 mM $MgCl_2$ and isolated after centrifugation at $90\,000 \times g$ for 30 min through 2.1 M sucrose, containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$. All steps were performed on ice or at 4°C.

Purity of the cytosolic, mitochondrial, microsomal and nuclear fractions was then tested, using specific enzyme markers, i.e. lactate dehydrogenase (LDH), cytochrome *c* oxidase, IDPase, NADH- and NADPH-cytochrome *c* reductase, acid phosphatase and UDP-galactosyltransferase, respectively, as well as Western blotting using compartment-specific antibodies directed against lamin B, PARP and adenylate kinases, as described previously.[19]

Western blot analysis

The loading was based on a constant protein concentration. Laemmli's loading buffer was added to the samples and after boiling for 4 min, the polypeptides were resolved on a 12% SDS gel at 130 V. The gels were transblotted onto nitrocellulose membranes (0.2 μm) for 2 h at 100 V. The membranes were blocked overnight in a buffer (50 mM Tris, pH 7.5, 500 mM NaCl) containing 1% bovine serum albumin and 5% non-fat milk powder. Then they were probed with primary antibodies in blocking solution without milk, followed by 1 h with secondary antibody in an identical solution, and visualized by ECL according to the manufacturer's instructions.

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