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PROCASPASE-9 IS ATTACHED TO THE MITOCHONDRIAL OUTER MEMBRANE IN THE EARLY STAGES OF APOPTOSIS

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Abstract: Procaspase-9 is the zymogen form of one of the apoptosis initiators, caspase-9. Its cellular location may differ depending on the cell type; it is found throughout the cytosol, although some of it may be associated with the mitochondria. Procaspase-9 relocates from the cytosol to the mitochondria shortly after the triggering of apoptosis in rat hepatocytes. We investigated whether the mitochondrial protein import machineries import procaspase-9. The combined results of protein import analyses, mitochondrial fractionation and protease treatments of intact and swollen mitochondria imply that procaspase-9 attaches to the outer surface of the mitochondrial outer membrane.

Key words: Caspase-9, Procaspase-9, Mitochondria, Apoptosis, Protein import, Rotenone, Localization

INTRODUCTION

Apoptosis or programmed cell death is an orderly process by which unwanted cells are dismantled [1]. Caspases are proteases that are central to the triggering and execution of apoptosis [2, 3]. Caspase-9 is one of the main initiators [4]. Like the other members of the caspase family, caspase-9 is present in the normal cell as an inactive zymogen (procaspase-9). It is activated by apoptotic stimuli

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Abbreviations used: DHFR – dihidrofolate-reductase; MPP – mitochondrial processing peptidase; OXA – oxidase assembly complex; PK – proteinase K; PMSF – phenylmethyl sulfonyl fluoride; pSu9 – targeting sequence of subunit 9 of F_0F_1-ATPase; SAM – sorting and assembly machinery; SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TIM – translocase of the inner membrane; TOB – topogenesis of the outer membrane; TOM – translocase of the outer membrane; Try – trypsin

that directly damage the mitochondria, or by signals originating in other parts of the cells [5-8].

Procaspase-9 is a cytosolic protein [9-14] that is partially associated with the mitochondria in some types of cells [15-19]. It was found to be in the cytosol of non-apoptotic pituitary cells, but to be redistributed to the mitochondria upon the initiation of apoptosis [14]. There are two main pathways for procaspase-9 activation: within the apoptosome, a large protein complex that consists of caspase-9, cytochrome c and Apaf-1 [9, 20, 21], or through proteolytic cleavage by a previously activated caspase, a process which involves its dimerization [6, 22, 23]. The formation of the apoptosome is thought to be the main pathway for the activation of procaspase-9. Apaf-1 was found only in the cytosol; therefore, the activation of procaspase-9 occurs mainly in the cytosol [9, 15, 19, 21]. To the best of our knowledge there has been no study on the mechanisms of transfer of procaspase-9 to the mitochondria, and particularly on whether it was only associated with the mitochondria due to transient interactions with mitochondrial proteins.

Mitochondrial proteins synthesized in the cytosol enter the mitochondria through complexes located in both mitochondrial membranes. The proteins are imported first through a translocation channel of the mitochondrial outer membrane (the TOM complex, translocase of the outer membrane) [24], which can function by itself for a small subset of outer membrane proteins or with other protein import complexes in the outer or inner mitochondrial membranes. The majority of mitochondrial proteins are imported through one of the two main protein import complexes of the mitochondrial inner membrane: the TIM23 and TIM22 complexes [24-26]. Their names are derived from the term translocase of the inner membrane (TIM) and their designated number, which is the molecular mass of their main component. TIM22 and TIM23 were characterized in mammals and yeast [24-26]. Three other protein translocases are currently known for yeast: TOB or SAM (for topogenesis of the outer membrane, and sorting and assembly machinery, respectively), OXA (oxidase assembly complex), and the recently identified translocase component Mia40 or Tim40 [24, 26]. TOB mediates the import, integration and assembly of outer membrane β-barrel proteins. OXA inserts the proteins from the mitochondrial matrix into the mitochondrial inner membrane, while Mia40 assists in the import of small metal co-ordinating proteins of the intermembrane space.

Mitochondrial proteins synthesized in the cytosol have mitochondrial targeting sequences, which direct them to the receptors of the TOM complex. Many soluble precursor proteins carry amino-terminal presequences, which contain hydrophilic and positively charged amino acids. The presequences are cleaved off by the processing peptidase in the mitochondrial matrix (MPP) [27]. Such proteins are imported through the TOM and TIM23 complexes into either the mitochondrial matrix, the inner membrane or even into the intermembrane space. The eleven N-terminal amino-acid residues of procaspase-9 were predicted to represent a mitochondrial targeting sequence by the computer

program PSORT [18]. The TIM23 complex needs two energy supplies: the mitochondrial membrane potential and ATP. Hydrophobic proteins, like mitochondrial carriers and the core components of the TIM complex (e.g. Tim23), span the membrane several times and carry internal targeting sequences. They are imported through the TOM and TIM22 complexes; the latter needs the membrane potential to insert them into the inner membrane.

Here, we report on a different distribution of procaspase-9 in normal and apoptotic hepatocytes: (1) in the cytosol of non-apoptotic cells; and (2) in the cytosol as well as colocalized with mitochondria early after the induction of apoptosis. By contrast to mitochondrial proteins like cholesterol side-chain cleavage cytochrome P-450 (P-450_{SCC}) [28, 29] and Tim23, which were used as controls, procaspase-9 was neither imported into mitochondria prepared from yeast nor was it imported efficiently into rat hepatocyte mitochondria *in vitro*. The association of procaspase-9 with the mitochondria was unaffected by the membrane potential, and MPP did not cleave it *in vitro*. The solubilization pattern of the endogenous procaspase-9 differed from that of cytochrome c when the mitochondria were treated with digitonin. Therefore, procaspase-9 was not soluble in the mitochondrial intermembrane space. It was solubilized from intact mitochondria by Triton X-100 under conditions in which the integral membrane protein Tim23 was not. Moreover, it was digested completely by proteinase K added to intact mitochondria. Its resistance to digitonin solubilization implied that procaspase-9 was attached to the membrane. We conclude that procaspase-9 becomes associated with the outer surface of the mitochondrial outer membrane during the early stages of apoptosis.

MATERIALS AND METHODS

Cell cultures of primary rat hepatocytes

Primary cell cultures of hepatocytes were isolated from adult male rats (Wistar-Hannover, 200-300 g) as described by De Sousa *et al.* [30]. Cells for immunocytochemistry were placed on poly-L-Lysin-coated coverslips and incubated in a standard medium overnight, and then incubated with or without 150 μM rotenone for 2 hours and used for immunocytochemistry. The hepatocytes used for the subcellular fractionations and isolations of mitochondria were transferred into 75 ml culture flasks and incubated with or without rotenone as above.

Subcellular fractionations and isolations of mitochondria

The mitochondria from the baker's yeast strain D273-10B were isolated as described previously [31]. Subcellular fractionations and isolations of mitochondria from rat livers were prepared in a HEPES-based buffer [32, 33].

In vitro synthesis of precursor proteins and import into isolated mitochondria

The plasmids for the following precursor proteins were used: procaspase-9 in pBluescriptII SK+ [34]; cytochrome P-450scc in pGEM4 [28]; Tim23 [35];

79pSu9-DHFR; and 84pSu9-DHFR [36]. The precursor proteins were synthesized by coupled *in vitro* transcription and translation in rabbit reticulocyte lysate (Promega), and were labelled with [^{35}S]methionine [37]. The precursor proteins were digested with 3 mg/ml MPP [38] at 25ºC for 10 minutes in a buffer containing 1 M KCl, 100 mM HEPES pH 7.4, 10mM DTT, 10 mM $MnCl_2$ and 30% reticulocyte lysate with precursor proteins.

The import of precursors into the mitochondria was performed in an import buffer containing 3% bovine serum albumin, 500 mM sorbitol, 80 mM KCl, 10 mM magnesium acetate, 2 mM KH_2PO_4, 2.5 mM EDTA, 2.5 mM $MnCl_2$ and 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.2 (HEPES-KOH). A standard assay also contained 2.5 mM malate, 2.5 mM succinate, 5 mM NADH, 2.5 mM ATP, 10 mM phosphocreatine, 0.1 mg/mg creatine kinase, 1 mg/ml mitochondria, and 1-3% reticulocyte lysate with the radiolabeled precursor protein. After incubation at 25ºC as indicated, the samples were split into three aliquots and diluted into buffers containing: (1) 0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.2 (HS, -PK); (2) HS with 50 μg/ml proteinase K (+PK); and (3) 20 mM Hepes-KOH, pH 7.2, with 50 μg/ml proteinase K. All the samples were incubated on ice for 25 minutes, 1 mM phenylmethyl sulfonyl fluoride (PMSF) was added, and 5 minutes later, the mitochondria or mitoplasts were reisolated by centrifugation.

Immunoblotting and immunocytochemistry

Hundred micrograms of mitochondrial proteins were typically resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The following antibodies were used: anti-caspase-9 (Cell Signaling Technology, Inc. or Stratagene), anti-cytochrome c (Calbiochem), and anti-Tim23 (BD Biosciences). They were detected by luminescence using secondary goat anti-rabbit or goat anti-mouse antibodies conjugated to horse radish peroxidase (BioRad).

Immunocytochemical analyses were performed using the standard protocol as described by the suppliers using the same primary antibodies as for immunoblotting. The signal was detected by appropriate secondary antibodies conjugated to the fluorescent dyes Alexa Fluor 546 (red, Molecular Probes) and Alexa Fluor 488 (green, Molecular Probes). The cover slips were mounted with ProLong Antifade Kit (Invitrogen, Molecular Probes). For double staining, the two primary antibodies (raised in different species) were added sequentially. Non-specific labelling by antibodies was tested by staining the cells with fluorescent secondary antibodies only. The cells were visualized using a Leica TCS SP confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) with an oil immersion objective (40x magnification and numerical aperture 1.25).

Digitonin fractionation and protease treatment

Isolated mitochondria (5.6 mg/ml) were solubilized in a buffer containing 250 mM sucrose, 1 mM EDTA, 10 M MOPS, pH 7.2, 80 mM KCl, and increasing concentrations of digitonin, from 0.05% to 0.4% (w/v). The

mitochondria were also solubilized in 0.15% Triton X-100. The samples were treated on ice for 30 minutes, then the solubilized proteins were separated from the mitochondria by centrifugation and precipitated by 15% trichloroacetic acid. Protease treatment of the mitochondria with 100 μg/ml trypsin or 100 μg/ml proteinase K was carried out in buffers (HS or 20 mM HEPES-KOH, pH 7.2) on ice for 20 min and stopped by the addition of 1 mM PMSF. The mitochondrial pellet was washed in HS and the proteins were resolved by SDS-PAGE.

RESULTS

Procaspase-9 is in the cytosol of non-apoptotic hepatocytes

The subcellular location of procaspase-9 in rat hepatocytes was determined through subcellular fractionation and confocal microscopy (Fig. 1). The mitochondrial and cytosolic fractions were isolated from primary cultures of rat hepatocytes using differential and density gradient centrifugations. In non-apoptotic cells, procaspase-9 was present only in the cytosol. Two hours after triggering apoptosis by the addition of rotenone, a substantial part of the procaspase-9 was in the mitochondrial fraction (Fig. 1A). The translocation of

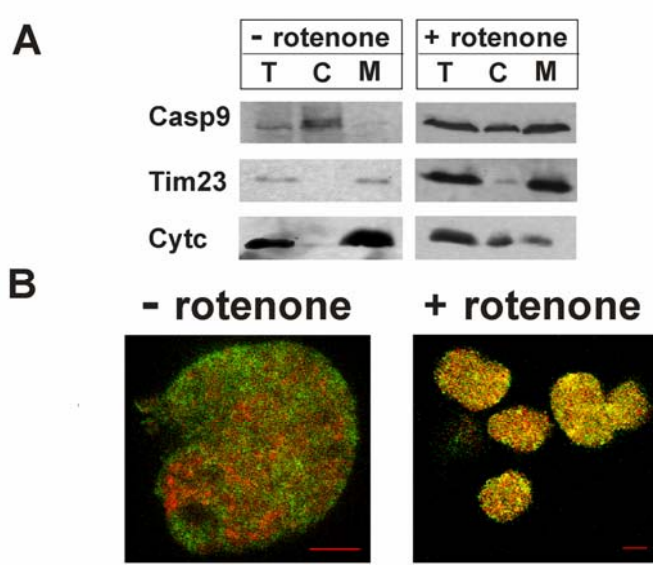

Fig. 1. Localization of procaspase-9 in normal (-rotenone) and apoptotic hepatocytes (+rotenone). A – Western blots of cell fractions; procaspase-9 (Casp-9), Tim23 and cytochrome c (Cytc) were detected from 100 μg of protein loaded per lane. T: total; C: cytoplasm; M: mitochondria. B. Immunocytochemistry of normal and apoptotic hepatocytes. Procaspase-9 (green), Tim23 (red). -Rotenone: bar 5 μm; +Rotenone: bar 10 μm.

procaspase-9 to the mitochondria was also observed in images of rat hepatocytes obtained by confocal fluorescence microscopy through fluorescent secondary antibodies against the caspase-9- and Tim23-specific antibodies (Fig. 1B). Therefore, procaspase-9 was in the cytoplasm of non-apoptotic hepatocytes and was redistributed to the mitochondria in the early phases of apoptosis.

Procaspase-9 is not imported into the interior of the mitochondria by the mitochondrial protein import machinery

Next, we investigated the ability of the mitochondria to import procaspase-9 *in vitro*. Procaspase-9 obtained by *in vitro* transcription and translation was incubated with mitochondria isolated from the rat liver (Fig. 2). Cytochrome P-450scc [28] and Tim23 [39] were used to control the import of proteins through the two inner membrane protein import complexes, TIM23 and TIM22, respectively. The imported precursors are shown on Fig. 2A. Compared to the import of cytochrome P-450scc and Tim23, considerably lower amounts of procaspase-9 were detected with energised rat liver mitochondria in import assays, even after 30-minute assays and long-term radioactive signal collection (Fig. 2A, +PK lanes). On exposures of autoradiographs normally needed to detect the imported proteins, the bands corresponding to procaspase-9 were barely visible, if at all (see also Fig. 2B, right panel). In addition, there was no need for the mitochondrial membrane potential, as seen by an equal low association of procaspase-9 in the absence of a membrane potential (Fig. 2B).

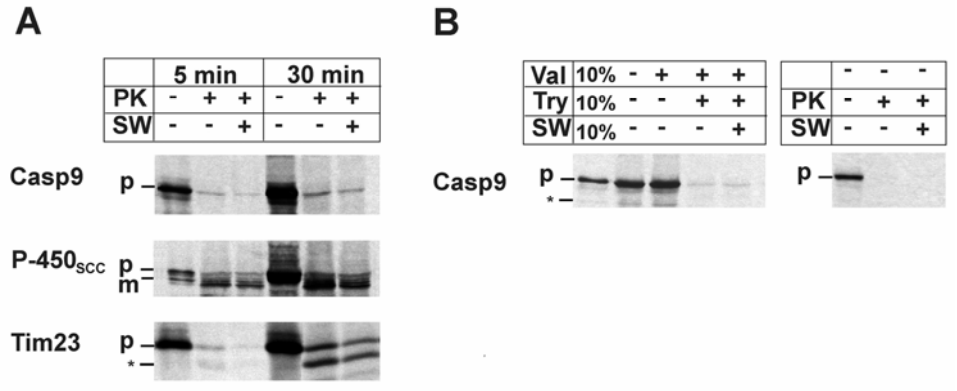

Fig. 2. Protein import into mitochondria isloated from non-apoptotic rat hepatocytes. Proteins synthesized by *in vitro* transcription and translation were added to mitochondria. To remove the unimported proteins, the mitochondria were treated after the import with trypsin (Try) or proteinase K (PK) in an isotonic buffer, or were swollen in a hypotonic buffer (SW). p: precursor; m: mature protein (without the targeting sequence). A – Import of procaspase-9 (Casp-9), cytochrome P-450$_{SCC}$ and Tim23. *: a portion of Tim23 clipped by PK. B – Import of procaspase-9 in the presence and absence of membrane potential. Membrane potential was dissipated by valinomycin (Val); procaspase-9 was imported for 30 minutes. *: the shorter translation product; 10%: 10% of the precursor, which was added to the mitochondria.

The hydrophobic transmembrane protein, Tim23, was imported with much higher efficiency. The minute amounts of protease-protected procaspase-9 likely arose from its undigested radioactive precursor. However, the import of small amounts of procaspase-9 cannot be excluded completely. For this, the residual amounts of procaspase-9 should be detected upon the opening of the mitochondria by detergents, which is very unlikely due to the losses during the experimental procedures.

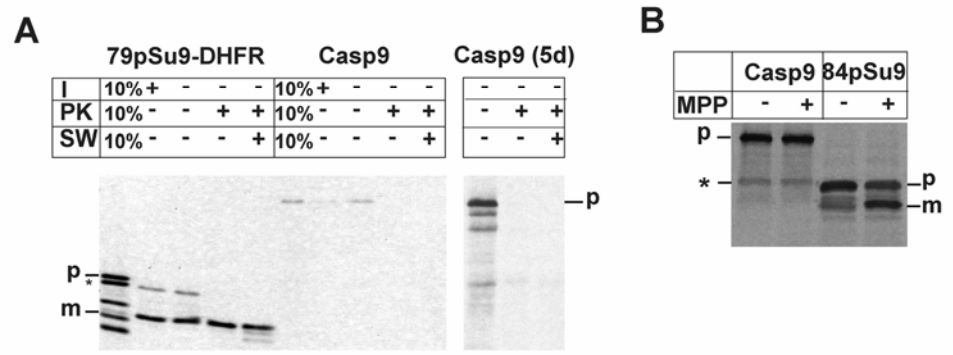

Fig. 3. Protein import into yeast mitochondria. p: precursor; m: mature protein (without the targeting sequence); 10%: 10% of the precursor added to the mitochondria. A – Mouse dihidrofolate reductase with 79 amino acid residues from subunit 9 of F_0F_1 ATPase of *N. crassa* forming its targeting sequence (79pSu9-DHFR) and procaspase-9 (Casp9) were imported for 30 minutes at 25ºC. After the import assay, mitochondria were either re-isolated (I), treated with proteinase K (PK), or swollen in a hypotonic buffer (SW). 5d: film exposure for 5 days; *: shorter translation products. B – Processing of targeting sequences by membrane processing peptidase (MPP). Dihidrofolate reductase with 84 amino acid residues from subunit 9 of F_0F_1 ATPase of *N. crassa* forming its targeting sequence (84pSu9-DHFR) and procaspase-9 synthesized by *in vitro* transcription and translation were digested by the isolated MPP. *: shorter translation products of procaspase-9.

Yeast mitochondria very efficiently import numerous proteins synthesized *in vitro*. We therefore tested them for the import of procaspase-9 (Fig. 3A). Unlike the murine dihidrofolate-reductase, carrying the targeting sequence of subunit 9 of F_0F_1-ATPase (pSu9-DHFR) [36], procaspase-9 was not imported into yeast mitochondria at all. Furthermore, it was not even bound tightly. It was lost upon re-isolation of the yeast mitochondria. Also, unlike the pSu9-DHFR, the procaspase-9 was not digested by the isolated membrane-processing peptidase *in vitro* (Fig. 3B). Our conclusion from all these experiments is that procaspase-9 is not imported into the mitochondria through the protein import machinery.

Procaspase-9 is associated with mitochondrial membranes

The submitochondrial localization of procaspase-9 in apoptotic hepatocytes was determined through fractionations. Mitochondria were treated with increasing

amounts of digitonin, from 0.05% to 0.4% (Fig. 4A). Procaspase-9 was more resistant to the digitonin treatment than the intermembrane protein cytochrome c, which was easily solubilized. The solubilisation pattern of procaspase-9 in the digitonin buffer was more similar to that of the transmembrane protein Tim23. However, when the mitochondria were solubilized with 0.15% of Triton-X100, procaspase-9 was completely extracted like cytochrome c, while Tim23 remained associated with the membrane (Fig. 4B). In summary, unlike cytochrome c, procaspase-9 is not located in the intermembrane space of mitochondria and is associated with the mitochondrial membrane.

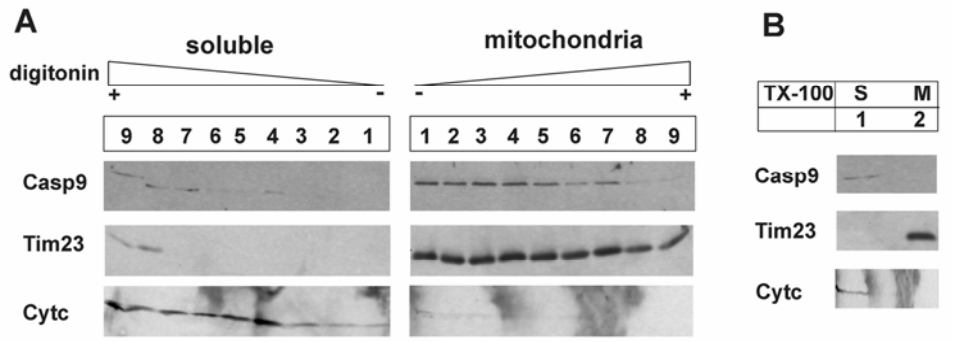

Fig. 4. Solubilization of mitochondria from rat apoptotic hepatocytes by detergents. A – Mitochondria incubated in 9 increasing concentrations of digitonin (lanes 9 to 1 and 1 to 9): from 0.05% (-) to 0.4% (+). The same number is designated to corresponding pellets and supernatants. The following concentrations of digitonin were used: 1 – 0.05%, 2 – 0.075%, 3 – 0.1%, 4 – 0.125%, 5 – 0.137%, 6 – 0.15%, 7 – 0.2%, 8 – 0.3%, 9 – 0.4%. B – Mitochondria were incubated in 0.15% Triton X-100 and solubilized proteins (S) were separated from the mitochondrial pellet (M).

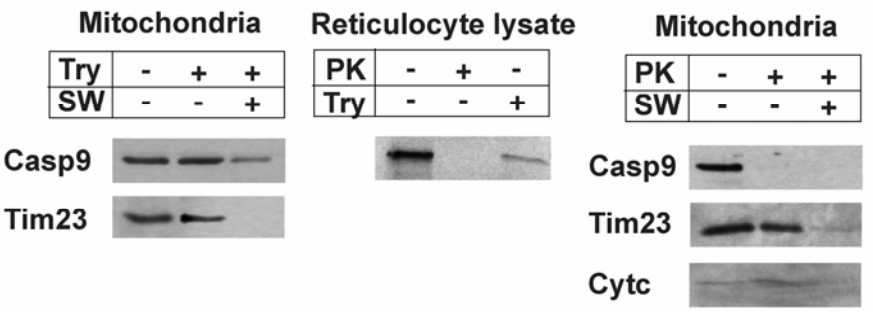

Fig. 5. Protease digests of rat apoptotic hepatocytes. Trypsin (Try) or proteinase K (PK) were added either to isolated mitochondria (Mitochondria) or to the precursor synthesized by *in vitro* transcription and translation (Reticulocyte lysate). The mitochondria were incubated in the hypotonic buffer, when indicated (SW).

Next, we performed protease digestion experiments to ascertain whether procaspase-9 was associated with the outer or inner mitochondrial membranes. We used trypsin and proteinase K (Fig. 5). Procaspase-9 was resistant to trypsin digestion even in swollen mitochondria, while Tim23 was not (Fig. 5, left panel). A similar pattern was observed upon trypsin digestion of procaspase-9, which was synthesized in the reticulocyte lysate (Fig. 5, middle panel). It is therefore likely that the minor amounts of procaspase-9 observed after treating mitochondria with trypsin resulted from incomplete digestion. By contrast, proteinase K completely digested the procaspase-9 synthesized in the reticulocyte lysate. Therefore, we used it to check the location of procaspase-9 detected with the mitochondria (Fig. 5, right panel). Procaspase-9 was degraded by proteinase K even when cytochrome c and Tim23 were protected. We conclude that procaspase-9 became associated with the mitochondria at the outer side of the mitochondrial outer membrane after the initiation of apoptosis by the addition of rotenone.

DISCUSSION

Here, we show that procaspase-9 is present in the cytoplasm of non-apoptotic hepatocytes and that about 50% of it is colocalized with the mitochondria shortly after the induction of apoptosis. This concurs with observations that procaspase-9 is distributed throughout the cytosol [9-14], and with those on its partial mitochondrial distribution [15-19]. The speed of accomplishing the invasive methods of cell isolation determined whether procaspase-9 was confined to the cytosol or was partially associated with the mitochondria. Therefore, the redistribution of procaspase-9 upon the induction of apoptosis in hepatocytes is a general process, not limited to the apoptosis triggered by rotenone.

The potential mitochondrial targeting sequence within the first 11 amino acid residues of procaspase-9 was previously predicted from the computer analysis of its sequence, but the import of procaspase-9 into the mitochondria was not tested [18]. The existence of the mitochondrial presequence implies that: (a) procaspase-9 is imported through the TIM23 complex; (b) at least its N-terminal protrudes into the mitochondrial matrix; and (c) the presequence is cleaved off by the membrane processing peptidase even if the protein is sorted subsequently into another mitochondrial subcompartment [27]. The presequence of procaspase-9 was not found to be removed in this study: neither during the *in vitro* import assays (unlike the P-450$_{SCC}$) nor when the precursor synthesized *in vitro* was treated with isolated MPP. In addition, negatively charged amino acid residues are typically not found in mitochondrial presequences; there are 3 such residues within the first 5 residues of human or rat procaspase-9. Therefore, procaspase-9 does not have a typical mitochondrial presequence, and it does not seem to be imported through the TIM23 complex. It is unlikely that any of the other mitochondrial protein import complexes transfer procaspase-9; even the extremely hydrophobic proteins with several membrane-spanning domains were

imported successfully, while the procaspase-9 was not. Also, yeast mitochondria should import mammalian proteins if these are equipped with proper mitochondrial targeting sequences, as in the case of mammalian frataxin [40]. Even mammalian mitochondria readily import the proteins derived from different tissues; P-450$_{SCC}$ that is normally expressed in the adrenal cortex is readily imported into rat liver mitochondria. In conclusion, procaspase-9 is not imported into the mitochondria through the mitochondrial protein import machinery. Interestingly, it is tightly bound to the rat liver mitochondria; however, not to those derived from yeast. This may imply the existence of a specific binding partner for procaspase-9 in the rat mitochondria.

Procaspase-9 was reported to reside in the intermembrane space, and in both the intermembrane space and in the mitochondrial outer membrane [16, 18, 19]. The differences in the observed locations of caspase-9 may arise due to the differences between the mitochondria derived from different types of cells, the cells being in different stages of apoptosis, or due to the differences within the experimental procedures. The latter case may apply to the results from immunogold electron microscopy of rat heart mitochondria, where 86% of procaspase-9 was reported in the intermembrane space, while 11% was associated with the surface of the mitochondria [18]. In the same study, the procaspase-9 was resistant to a trypsin digest; also, procaspase-9 and cytochrome c were solubilised by 1% digitonin. Unlike the former, the latter results are in complete agreement with ours. In our case, the procaspase-9 was not digested by trypsin even though we used up to 3-fold more trypsin compared to the above-mentioned study. Because of the inefficient digests of procaspase-9 by trypsin, we switched to proteinase K. The procaspase-9 was solublized completely in 0.2% digitonin, while all cytochrome c had already appeared in solution at 0.1% of digitonin; therefore, both proteins could be solubilised at much higher concentrations of digitonin as well. Chandra and Tang [19] deduced the location of caspase-9 to the intermembrane space based on the treatment of GM701 cells with 0.1 μg/ml proteinase K, while Yuan *et al.* [16] found that 50% of caspase-9 was degraded when Jurkat T-lymphocytes were treated with 50 μg/ml of proteinase K, and therefore reported that procaspase-9 is located at both sides of the mitochondrial outer membrane. The results presented here agree with both studies, since we managed to completely digest the procaspase-9 by the higher concentration of proteinase K (100 μg/ml). Unlike Chandra and Tang [19], we did not detect any active caspase-9. This is not surprising since our study focused exclusively on the early events after the triggering of apoptosis. Prolonged incubation with rotenone results in apoptotic cell death (I.M. unpublished results). The events around the activation of procaspase-9 remain to be investigated further.

In conclusion, our data concurs with all the data obtained via similar methods. The systematic mitochondrial fractionations through small increases of digitonin concentrations and rigorous protease digests have uncovered that the solubilisation pattern of procaspase-9 differs from that of cytochrome c and that

procaspase-9 is accessible to protease digestions in intact mitochondria. Therefore, procaspase-9 is attached to the outer surface of the mitochondrial membrane; either entirely or partially translocated across the outer membrane in a protease-unprotected manner. As it is a soluble protein, it is likely that procaspase-9 has a binding partner in the outer mitochondrial membrane. Future research should focus on discovering this binding partner and identifying the physiological role of the redistribution of procaspase-9 to the mitochondria after the triggering of apoptosis and before its irreversible activation through proteolytic processing.

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