Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization

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Mitochondrial cytochrome c, which functions as an electron carrier in the respiratory chain, translocates to the cytosol in cells undergoing apoptosis, where it participates in the activation of DEVD-specific caspases. The apoptosis inhibitors Bcl-2 or Bcl-xL prevent the efflux of cytochrome c from mitochondria. The mechanism responsible for the release of cytochrome c from mitochondria during apoptosis is unknown. Here, we report that cytochrome c release from mitochondria is an early event in the apoptotic process induced by UVB irradiation or staurosporine treatment in CEM or HeLa cells, preceding or at the time of DEVD-specific caspase activation and substrate cleavage. A reduction in mitochondrial transmembrane potential (ΔΨm) occurred considerably later than cytochrome c translocation and caspase activation, and was not necessary for DNA fragmentation. Although zVAD-fmk substantially blocked caspase activity, a reduction in ΔΨm and cell death, it failed to prevent the passage of cytochrome c from mitochondria to the cytosol. Thus the translocation of cytochrome c from mitochondria to cytosol does not require a mitochondrial transmembrane depolarization.

Keywords: apoptosis/caspases/cytochrome c/ΔΨm/
mitochondria

Introduction

Apoptotic cell death is a fundamental feature of virtually all animal cells (Jacobson et al., 1997). It is an indispensable process during normal development, tissue homeostasis, development of the nervous system and the regulation of the immune system. Insufficient or excessive cell death can contribute to human disease, including cancer or degenerative disorders (Thompson, 1995). Irrespective of the cell death-inducing signal, cells die in a highly coordinated and stereotyped manner, with 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 common death program, towards which diverse signal-transducing pathways converge (White, 1996; Chinnaiyan and Dixit, 1996; Vaux and Strasser, 1996).

Apoptosis is genetically regulated, as best demonstrated by studies in the nematode Caenorhabditis elegans (Ellis et al., 1991; Horvitz et al., 1994). In this organism, developmental cell death involves several regulatory genes, including ced-3, ced-4 and ced-9. CED-3 and CED-4 are required for cell death to occur, whereas CED-9 inhibits cell death (Hengartner et al., 1992). CED-9 shares sequence and functional similarity with the mammalian cell death inhibitor molecule, Bcl-2 (Hengartner and Horvitz, 1994). Recent biochemical data indicate that CED-4, CED-3 and CED-9 can physically interact (Chinnaiyan et al., 1997; James et al., 1997; Spector et al., 1997; Wu et al., 1997). The ced-3 gene encodes a cysteine protease and shares sequence similarity with a family of at least 10 mammalian cysteine proteases with aspartate specificity, now termed the caspases (Alnemri et al., 1996), or formerly the ICE (interleukin-1β-converting enzyme) family (Yuan et al., 1993; Martin and Green, 1995; Chinnaiyan and Dixit, 1996; Fraser and Evan, 1996; Henkert, 1996; Patel et al., 1996; Nagata, 1997). Each caspase is synthesized as an inactive cytosolic precursor molecule, and in cells induced to undergo apoptosis is converted by proteolytic cleavage to an active heterodimer composed of two chains of ~20 and 10 kDa. The molecular mechanism by which the caspases become activated in the apoptotic process is currently not known. The mammalian caspase-3 (formerly called CPP32/apopain/Yama) shares most sequence similarity and substrate specificity with the C.elegans death protease CED-3, and has been shown in vitro and in vivo to play a critical role in the apoptotic process (Miura et al., 1993; Yuan et al., 1993; Kuida et al., 1996; Xue et al., 1996).

Several substrates have been identified that are cleaved by caspase-3 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), sterol-regulatory element-binding protein (SREBPs) (X.Wang et al., 1996), the U1-associated 70 kDa protein (Casciola-Rosen et al., 1996) D4-GDI (Na et al., 1996), huntingtin (Goldberg et al., 1996), DNA-dependent protein kinase (Casciola-Rosen et al., 1996; Song et al., 1996), MEKK (Cardone et al., 1997) and the DNA fragmentation factor (DFF) (Liu et al., 1997). Cleavage of specific substrates has been proposed to either activate death effector molecules or trigger the structural changes characteristic of apoptotic cells.

Recent evidence is emerging that mitochondria participate in the central control or executioner phase of the cell death cascade (Newmeyer et al., 1994; Ankarcrona et al., 1995, Castedo et al., 1995; Cossarizza et al., 1995; Petit et al., 1995; Zamzami et al., 1995a,b, 1996a,b; Henkart and Grinstein, 1996; Krippner et al., 1996; Liu et al., 1996; Murphy et al., 1996; Polla et al., 1996; Susin et al., 1996, 1997; Chinnaiyan et al., 1997; Golstein, 1997; James et al., 1997; Kroemer, 1997a,b; Kroemer et al., 1998; Punスキ和et al., 1997).
whether a reduction in $\Delta \Psi_m$ could account for the mitochondrial efflux of cytochrome c in apoptotic cells. In addition, we investigated whether active caspases might be required for the release of cytochrome c from mitochondria. Data presented here indicate that passage of cytochrome c from mitochondria to cytosol occurs early in the apoptotic process, and can proceed independently of DEVD-specific caspase activity and a reduction in $\Delta \Psi_m$.

**Results**

**Mitochondrial release of cytochrome c occurs before or at the time of DEVD-specific caspase activation and a reduction in $\Delta \Psi_m$**

To examine the sequence of events that link mitochondrial cytochrome c release to other molecular events in the cell death process, cytochrome c release, caspase activity and $\Delta \Psi_m$ were monitored simultaneously over several hours after apoptosis induction. The human T lymphoblastoid cell line, CEM, was treated with the protein kinase inhibitor staurosporine, an inducer of apoptosis in most cell types (Jacobson et al., 1996; Weil et al., 1996) and cytosolic extracts were prepared at various times, under conditions that keep mitochondria intact. Cytosolic cytochrome c protein levels were measured by immunoblot analysis. Cytosol from untreated CEM cells did not contain any detectable cytochrome c protein (Figure 1A). In contrast, cytosolic cytochrome c accumulated significantly at 1–2 h of treatment with staurosporine (Figure 1A). At 4 h, cytochrome c was maximally released from mitochondria and no further increase was observed thereafter. The absence of cytochrome oxidase (subunit II) in cytosolic extracts confirmed that our preparations were free of mitochondrial contamination. Furthermore, cytochrome c release preceded maximal apoptosis as measured by binding of fluorescein isothiocyanate (FITC)-conjugated annexin V to externalized phosphatidylserine, a relatively early event in apoptotic cell death (Martin et al., 1995).

Proteolytic activity associated with caspases was measured in three different ways. First, the cytosolic extracts were tested for their ability to cleave the colorimetric peptide substrate DEVD-pNA, which is recognized by several caspases. DEVD-pNA cleavage activity started to increase at 2–3 h after staurosporine treatment (Figure 1B). After 6 h, no further increase in DEVD-pNA cleavage activity could be detected (Figure 1B). Second, the cleavage of an endogenous caspase substrate, PARP, was examined by immunoblot analysis and densitometric scanning of the PARP cleavage product. The PARP cleavage product was detectable at 2–3 h after treatment with staurosporine (Figure 1B). Third, pro-caspase-3 activation was examined by immunoblot analysis. Similar to results obtained by measuring DEVD-pNA and PARP cleavage, pro-caspase-3 became proteolytically activated at 2–3 h following treatment with staurosporine (Figure 1B). In summary, caspases were activated at 2–3 h after staurosporine treatment, thus following the export of cytochrome c from mitochondria.

A reduction in $\Delta \Psi_m$ accompanies early apoptosis in many experimental systems, believed to be mediated by the opening of the mitochondrial PTP, a multi-protein complex (reviewed in Zarotti and Szabo, 1995; Bernardi and Petronilli, 1996; Zamzami et al., 1996a,b; Kroemer, 1997a; Kroemer et al., 1997). We therefore examined
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Mitochondrial cytochrome c enters the cytosol prior to activation of DEVD-specific caspases and a reduction in ΔΨₘ in CEM cells induced to undergo apoptosis by staurosporine treatment (1 μM). (A) Cytosolic extracts were prepared at the indicated times of staurosporine treatment (1 μM), and cytochrome c (Cyt.c), cytochrome c oxidase (subunit II) (Cyt.c ox. II) and actin were evaluated by immunoblot analysis. Cyt.c ox.II served as an indicator for mitochondrial contamination of cytosolic extracts. Actin was used as a control for protein loading. The percentage of apoptosis was determined by annexin-V–FITC binding. A fraction of isolated mouse liver mitochondria (Mit.fr.) was analyzed as a positive control for cytochrome c and cytochrome c oxidase (subunit II). The release of cytochrome c was quantified by densitometric scanning of the autoradiogram film and plotted against time in hours after staurosporine treatment. (B) Activation of DEVD-specific caspases was measured using the colorimetric substrate DEVD-pNA. Activation of pro-caspase-3 and cleavage of PARP were analyzed by immunoblotting analysis. Pro-caspase-3 activation is seen as a loss of its pro-form. The PARP cleavage product is indicated by an arrow. The increase in PARP cleavage product was quantified by densitometric scanning of the autoradiogram film and the values were plotted against time after staurosporine treatment. (C) Measurement of ΔΨₘ by CMTMRos staining and flow cytometry. The percentage of cells with low ΔΨₘ is seen as a shift to weaker CMTMRos fluorescence and was quantitated using CellQuest software. The percentage of cells with a low ΔΨₘ was plotted against time after staurosporine treatment.

To investigate whether a reduction in ΔΨₘ could explain the mitochondrial cytochrome c efflux in apoptosis, ΔΨₘ was monitored by fluorescence of the cationic lipophilic dye CMTMRos, a potential-sensitive dye (Macho et al., 1996). CMTMRos dye uptake remained essentially unaltered during the first 4 h after staurosporine treatment (Figure 1C), a time at which cytochrome c had been maximally released from mitochondria and caspases were active (Figure 1A and B). A moderate reduction in ΔΨₘ was detected 5 h after exposure to staurosporine as measured by a decrease in CMTMRos fluorescence (Figure 1C). To confirm that the CMTMRos dye was sensitive to mitochondrial transmembrane depolarization, control CEM cells were treated with the mitochondrial uncoupling agent carbonyl cyanide m-chlorophenylhydrazone (mCICCP) (50 μM). After 15 min, the entire cell population exhibited a shift to weaker fluorescence, indicative of a reduction in ΔΨₘ, reminiscent of the decrease in fluorescence observed in apoptotic cells (data not shown).

To extend these findings to another apoptotic stimulus, CEM cells were also exposed to UVB light (36 J/cm²). Similar results were obtained as for staurosporine-treated CEM cells, in that cytosolic cytochrome c began to accumulate by 2 h and increased until 5 h after radiation exposure (Figure 2A). Cytochrome c oxidase (subunit II) protein was undetectable in all cytosolic extracts, confirming that the presence of cytochrome c protein was not a simple reflection of a contamination of extracts with mitochondria. As for staurosporine-treated CEM cells, cytochrome c preceded the onset of apoptosis, as measured by annexin-V–FITC binding to cell surface phosphatidylserine (Figure 2A).

When caspase activity was assayed by cleavage of the peptide substrate DEVD-pNA in UVB-treated CEM cells, cleavage activity was detectable 2–3 h after radiation...
Fig. 2. Cytochrome c accumulates in the cytosol of CEM cells induced to undergo apoptosis by UVB irradiation (36 J/cm²), when DEVD-specific caspases become activated and prior to ΔΨₘ decrease. (A) Cytosolic cytochrome c and percentage apoptosis were assessed as in Figure 1A. (B) Caspase activation and PARP cleavage were assessed as in Figure 1B. (C) ΔΨₘ was assessed as in Figure 1C.

tion exposure. The PARP cleavage product was detected slightly earlier than DEVD-pNA cleavage activity, at 2 h following exposure to UVB light as quantified by densitometric scanning of the immunoblot (Figure 2B). A loss of pro-caspase-3 was detected at 2–3 h after UVB treatment. Thus in UVB-induced apoptosis of CEM cells, cytochrome c release occurred at approximately the same time as the activation of DEVD-specific caspases.

A decrease in ΔΨₘ was detected at 3 h in UVB-treated CEM cells (Figure 2C). While a decrease in ΔΨₘ was observed earlier in UVB- than in staurosporine-induced cell death, it nevertheless occurred after cytochrome c release from mitochondria.

In summary, these results show that cytochrome c release from mitochondria is an early event in the apoptotic process in CEM cells, occurring before or at the time of DEVD-specific caspase activation and preceding a decrease in ΔΨₘ.

A reduction in ΔΨₘ is not required for DNA fragmentation in staurosporine-induced apoptosis

It has been proposed that a decrease in ΔΨₘ is the first step in the apoptotic process, when cells become irreversibly committed to die, prior to DNA fragmentation (Zamzami et al., 1995b; Macho et al., 1996). However, data presented in Figures 1C and 2C indicate that a decrease in ΔΨₘ is a relatively late event in the cell death process induced in CEM cells by staurosporine or UVB light. This raised the possibility that apoptosis may not necessarily require a reduction in ΔΨₘ to proceed.

We therefore examined apoptosis in HeLa cells in which DNA fragmentation and ΔΨₘ can be examined visually by confocal microscopy. As in CEM cells, HeLa cells released cytochrome c into the cytosol following exposure to UVB light (Figure 3A). Similar results were obtained with staurosporine-treated HeLa cells (data not shown). An increase in cytosolic cytochrome c correlated with activation of caspases as measured by cleavage of the synthetic peptide substrate DEVD-pNA or the endogenous substrate PARP (Figure 3A and B).

ΔΨₘ and DNA fragmentation were examined simultaneously in individual HeLa cells induced to undergo apoptosis by staurosporine (1 μM) or UVB (14 J/cm²) treatment. At 5 h post-treatment, cells were first loaded with the dye CMTMRos to measure ΔΨₘ and then fixed to stain with the TUNEL technique to monitor DNA fragmentation. As shown in Figure 3C, untreated HeLa cells exhibited a normal mitochondrial membrane poten-
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Fig. 3. (A) Mitochondrial cytochrome \( c \) accumulates in the cytosol of HeLa cells induced to undergo apoptosis by UVB irradiation (36 J/cm\(^2\)) and correlates with DEVD-specific caspase activation. Cytosolic extracts were prepared at the indicated times after exposure to UVB light, and cytochrome \( c \), pro-caspase-3, PARP and actin were detected by immunoblotting. The percentage of cells in apoptosis was determined by eosin and hematoxylin staining and microscopic assessment. (B) Caspase activity of the samples in (A) was measured by cleavage of the colorimetric substrate DEVD-pNA. The cleavage activity over time after UVB irradiation is shown. The increase in the PARP cleavage product was quantified by densitometric scanning of the PARP immunoblot of (A) and values are plotted against time after UVB irradiation. (C) A reduction in \( \Delta \Psi_m \) is not necessary for DNA fragmentation. HeLa cells were induced to undergo apoptosis by treatment with staurosporine (1 \( \mu \)M) or UVB light (14 J/cm\(^2\)), and analyzed 5 h later. \( \Delta \Psi_m \) (bright red, mitochondrial staining) and DNA fragmentation (green, nuclear staining) were determined simultaneously by staining with CMTMRos and the TUNEL technique. Shown are untreated HeLa cells (top panel), staurosporine-treated HeLa cells (second panel from top), the major population of UVB-irradiated HeLa cells (third panel from top) and a minor population of UVB-irradiated HeLa cells (bottom panel). The bar is 10 \( \mu \)m.

A decline in intracellular ATP levels follows the early phase of apoptosis

To examine how quickly a loss of mitochondrial cytochrome \( c \) would lead to a decrease in cellular energy metabolism, CEM cells were treated with staurosporine (1 \( \mu \)M), which consistently induced early cytochrome \( c \) translocation, and changes in intracellular ATP levels were measured. As shown in Figure 4, the intracellular ATP concentration remained initially constant, but declined considerably after 6 h of treatment with staurosporine. Similar results were also obtained for UVB-treated CEM cells (data not shown). Thus, the intracellular ATP pool appeared to be unaltered in the early phase of apoptosis, but declined substantially after cytochrome \( c \) was lost from the mitochondria, and the DEVD-specific caspases were active (Figures 1, 2 and 4).

A caspase inhibitor, zVAD-fmk, prevents the decrease in \( \Delta \Psi_m \) following UVB irradiation

A decrease in \( \Delta \Psi_m \) was observed following UVB or staurosporine treatment after mitochondrial release of cytochrome \( c \) and after or at the time of caspase activation (Figures 1 and 2). Therefore, it was possible that active caspases may induce a decrease in \( \Delta \Psi_m \). To test this possibility, CEM cells were either left untreated or pretreated for 1 h with z-Val-Ala-Asp-CH\(_2\)F (zVAD-fmk), a cell-permeable, irreversible caspase inhibitor of broad specificity (Nicholson et al., 1995; Armstrong et al., 1996),
and apoptosis was induced by exposure to UVB light. \( \Delta \Psi_m \) was measured by the uptake of the fluorochrome CMTMRos over several hours. A decline in \( \Delta \Psi_m \) was observed at 3 h after UVB irradiation and, at 8 h, the entire cell population exhibited a complete loss of \( \Delta \Psi_m \) (Figure 5A). In contrast, zVAD-fmk markedly reduced the loss of \( \Delta \Psi_m \) (Figure 5B). However, a very minor shift to weaker fluorescence was noticed in zVAD-fmk plus UVB-treated CEM cells (Figure 5B). However, this small decrease was not detectable until at least 3 h post-treatment, well after the release of cytochrome c, which can be observed consistently in these cells (Figure 2A). These data suggest that activated caspases impinge on mitochondria to induce mitochondrial permeability transition (PT) and a subsequent loss in \( \Delta \Psi_m \) during apoptosis provoked by UVB irradiation. Similar findings have been reported recently in Fas-induced apoptosis, in which caspase inhibitors were able to block PT-induced \( \Delta \Psi_m \) loss (Boise and Thompson, 1997; Susin et al., 1997).

**Caspase inhibition has no effect on the translocation of cytochrome c from mitochondria to cytosol**

Since the \( \Delta \Psi_m \) loss was effectively blocked by the caspase inhibitor zVAD-fmk, we tested whether the relocation of cytochrome c from mitochondria to the cytosol would also be dependent on active caspases. CEM cells were either left untreated or pre-incubated with zVAD-fmk and then induced to undergo apoptosis by exposure to UVB light (36 J/cm²) or staurosporine (500 nM). At 6 h post-treatment, cytosolic extracts were obtained and cytochrome c, cytochrome c oxidase (subunit II), pro-caspase-3, PARP and actin protein levels were evaluated by immunoblot analysis. As shown in Figure 6A, pre-treatment of cells with zVAD-fmk had no effect on the cytosolic accumulation of cytochrome c in UVB- or staurosporine-treated CEM cells, yet the inhibitor effectively suppressed pro-caspase-3 processing, PARP cleavage, loss of \( \Delta \Psi_m \) and cell death. In addition, zVAD-fmk completely blocked the appearance and/or function of active caspases capable of cleaving DEVD-pNA (Figure 6B). Thus active caspases are not required for the mitochondrial release of cytochrome c in UVB- or staurosporine-induced cell death. In contrast, active caspases seem to induce PT, measured by a reduction in \( \Delta \Psi_m \) as the caspase inhibitor zVAD-fmk substantially blocked these mitochondrial events (Figures 5 and 6A). Thus, a disruption in \( \Delta \Psi_m \) is unlikely to be the mechanism responsible for the release of cytochrome c.

To confirm the finding that cytochrome c release can proceed independently of caspase activation or function, the cellular localization of cytochrome c protein was examined by confocal immunofluorescence microscopy in HeLa cells. In untreated HeLa cells, cytochrome c displayed punctate cytoplasmic staining, which is in agreement with its localization in mitochondria (Figure 7A). After apoptosis induction by UVB irradiation, apoptotic cells, identified by chromatin condensation, nuclear fragmentation and cytosolic vacuolization, exhibited mostly diffuse cytoplasmic cytochrome c staining, which is consistent with its translocation from mitochondria to cytoplasm (Figure 7C). In UVB-treated HeLa cells overexpressing Bcl-2, cytochrome c displayed primarily a mitochondrial immunostaining pattern, similar to HeLa control cells (Figure 7B), confirming previous observations that Bcl-2 or Bcl-xL block the release of cytochrome c from mitochondria (Kim et al., 1997; Kluck et al., 1997a; Yang et al., 1997). While zVAD-fmk protected cells from...
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Fig. 6. Cytochrome c release does not require active caspases and a reduction in ΔΨm. (A) CEM cells were treated with zVAD-fmk (1 h, 100 μM) and then induced to undergo apoptosis by UVB irradiation (36 J/cm²) or by staurosporine treatment (500 nM) and were analyzed after 6 h. Cytochrome c (Cyt.c), cytochrome c oxidase subunit II (Cyt.c ox. II), pro-caspase-3, PARP and actin protein levels were determined by immunoblotting. The PARP cleavage product is indicated by the arrow. Caspase-3 activation is represented by the loss of its pro-form. The percentage of cells with low ΔΨm was measured by staining with CMTMRos (150 nM) and flow cytometry and quantified by CellQuest software. The percentage apoptosis was determined by annexin-V–FITC binding. (B) zVAD-fmk effectively blocks DEVD-specific cleavage activity in cells treated with UVB light or staurosporine. Caspase activity of cytosolic extracts analyzed in (A) was measured using the colorimetric substrate DEVD-pNA.

Discussion

In this report, we show that the release of cytochrome c from mitochondria to cytosol is an early event in the apoptotic process, preceding morphological signs of apoptosis. After or at the time of the mitochondrial cytochrome c efflux, the effector caspases of DEVD specificity, including pro-caspase-3, become activated and PARP is cleaved. This is in accord with the notion that cytochrome c may be required for caspase activation during the induction of apoptosis, at least in some situations (Liu et al., 1996; Kluck et al., 1997a,b; Vaux, 1997; Yang et al., 1997; Zou et al., 1997; S.J. Martin, R.M. Kluck, C.A. Castiano, D.D. Newmeyer, H.-G. Wang, J.C. Reed, D.D. Nicholson, E.S. Alnemri and D.R. Green, in preparation). Thus, while our results do not establish a connection between cytochrome c release and subsequent caspase activation in intact cells, they are consistent with observations made in cell-free systems.

Cytochrome c was released from coupled mitochondria, since a reduction in ΔΨm was observed only later in the process (Figures 1 and 2). A breakdown in ΔΨm was not found until after DNA fragmentation in HeLa cells induced to undergo apoptosis by staurosporine treatment, indicating that mitochondrial transmembrane depolarization is not required for apoptosis under some circumstances. A similar late decrease in ΔΨm was also found in several other instances of apoptosis (Deckwerth and Johnson, 1993; Ankarcrona et al., 1995; Cossarizza et al., 1995; Tropea et al., 1995). However, several reports described an early ΔΨm decrease with apoptosis, prior to the exposure of phosphatidylserine on the cell surface or nuclear DNA fragmentation (Zamzami et al., 1995a,b; Castedo et al., 1995). Furthermore, isolated cells with low ΔΨm underwent DNA fragmentation even when the apoptosis-inducing stimulus was removed, suggesting that ΔΨm loss may irreversibly commit cells to apoptosis (Zamzami et al., 1995b). This discrepancy may be due to differences in cell types or apoptosis-inducing stimuli. Our results do not exclude the possibility that changes in ΔΨm are critical for apoptosis in some cases, but support the idea that this is not a universal step in the apoptotic process.

Mechanism of cytochrome c translocation

The molecular mechanism responsible for the translocation of cytochrome c from mitochondria to cytosol is at present unknown. As previously shown, diverse apoptosis-inducing agents, including UVB light, staurosporine, etoposide, actinomycin D, hydrogen peroxide, ara-C and ligation of the Fas receptor, are capable of provoking the release of cytochrome c from mitochondria.
Fig. 7. Cytochrome c release visualized by confocal immunofluorescence microscopy. Immunolocalization of cytochrome c (green) and nuclear morphology (red) is shown in untreated HeLa cells (A); in UVB-irradiated HeLa cells with Bcl-2 overexpression (B); in UVB-irradiated HeLa cells (C); and in HeLa cells pre-incubated with zVAD-fmk (100 μM, 1 h) and then irradiated with UVB light (D). Cells were grown for 24 h on glass coverslips and treated with UVB light (14 J/cm²), fixed 4 h after treatment and immunostained with anti-cytochrome c monoclonal antibodies (clone 7H8C12). Chromatin was stained with propidium iodide. The bar is 20 μm.

(Krippner et al., 1996; Liu et al., 1996; Kim et al., 1997; Kluck et al., 1997a; Yang et al., 1997). Most, if not all cytochrome c enters the cytosol after apoptosis induction (Kim et al., 1997; Yang et al., 1997; E.Bossy-Wetzel, unpublished results) Mitochondrial cytochrome c release may therefore be a common event in the cell death effector pathway, initiated by diverse signal-transducing events. If cytochrome c is then responsible for triggering caspase activation and subsequent caspase-dependent apoptotic events, as suggested by in vitro studies (Kluck et al., 1997a,b; Liu et al., 1997; Zou et al., 1997; S.J.Martin, R.M.Kluck, C.A.Casiano, D.D.Newmeyer, H.-G.Wang, J.C.Reed, D.D.Nicholson, E.S.Alnemri and D.R.Green, in preparation), then the release of cytochrome c from mitochondria is a central event in apoptosis. However, further investigations are required to determine whether caspase activation in vivo is dependent on cytosolic cytochrome c.

A decrease in ΔΨm has been reported to accompany apoptosis induced by diverse stimuli and in different cell types, and Bcl-2 or Bcl-xL block these mitochondrial events (Deckwerth and Johnson, 1993; Vayssiere et al., 1994; Ankararona et al., 1995; Castedo et al., 1995; Cossarizza et al., 1995; Petit et al., 1995; Zamzami et al., 1995a,b, 1996a,b; Shimizu et al., 1996b). This report shows that cytochrome c can be released from mitochondria which still maintained a normal and high ΔΨm in that a loss in ΔΨm was not observed until considerably after cytochrome c export and activation of DEVD-specific caspases (Figures 1 and 2). Consequently, translocation of cytochrome c from mitochondria does not rely on a reduction in ΔΨm. This is in agreement with previous reports in which ΔΨm remained stable in staurosporine- or ara-C-treated HL-60 cells for several hours after cytochrome c was released from mitochondria (Kim et al., 1997; Yang et al., 1997). Moreover, the caspase inhibitor zVAD-fmk effectively blocked the reduction in ΔΨm induced by apoptosis-provoking agents, but failed to block the release of cytochrome c. This suggests that a fall in ΔΨm may be a consequence of caspase activity rather than the effector mechanism driving cytochrome c efflux. Similarly, a reduction in ΔΨm has been reported to be blocked by zVAD-fmk in Fas-induced apoptosis (Boise and Thompson, 1997; Susin et al., 1997). However, in this situation, inhibitors may be acting primarily on the upstream caspases such as caspase-8 (Boldin et al., 1996; Muzio et al., 1996). Finally, we previously showed that treatment of isolated mitochondria with the protonophore and uncoupler mCICCP led to a rapid decrease in ΔΨm, yet failed to elicit cytochrome c release and apoptosis (Kluck et al., 1997a). Thus a reduction in ΔΨm per se is not sufficient to permit cytochrome c translocation.

This is not to exclude the possibility that in some cases a PT which is associated with the opening of the so-called mitochondrial megapore, a multi-protein complex, may be implicated in the release of cytochrome c (Zarotti and Szabo, 1995; Bernardi and Petronilli, 1996). In recent studies by Kroemer and colleagues, isolated mitochondria were induced to undergo PT and a subsequent reduction in ΔΨm, and liberated a 50 kDa protease, termed
apoptosis-inducing factor (AIF), that was sufficient to induce apoptotic changes in isolated nuclei without additional cytoplasmic components (Susin et al., 1996, 1997; Zamzami et al., 1996a,b). Furthermore, Bcl-2 prevented mitochondrial membrane depolarization and release of AIF. As shown recently, the PTP can operate in two modes: a high conductance state, which is associated with a persistent pore opening and results in a long-term reduction in ΔΨₘ, and a low conductance state, which is associated with a transitory pore opening and a mitochondrial depolarization spike (Ichas et al. 1997). It can be speculated that the entry of cytochrome c into the cytosol may not require a complete irreversible reduction in ΔΨₘ, but may be related to a transitory opening of the PTP, operating in a low conductance mode. In agreement with this idea is the observation that treatment of isolated rat liver mitochondria with atracyloside (5 mM) or calcium chloride (100 μM), two inducers of PT, triggers the release of cytochrome c in vitro (Kantrow and Piantadosi, 1997). Cyclophilin A (CsA), a ligand of the mitochondrial matrix cyclophilin D, can inhibit PT and the pre-apoptotic mitochondrial depolarization spike (Ichas et al. 1997). It can be speculated that the entry of cytochrome c into the cytosol may not require a complete irreversible reduction in ΔΨₘ, but may be related to a transitory opening of the PTP, operating in a low conductance mode. In agreement with this idea is the observation that treatment of isolated rat liver mitochondria with atracyloside (5 mM) or calcium chloride (100 μM), two inducers of PT, triggers the release of cytochrome c in vitro (Kantrow and Piantadosi, 1997). Cyclophilin A (CsA), a ligand of the mitochondrial matrix cyclophilin D, can inhibit PT and the pre-apoptotic mitochondrial depolarization spike (Ichas et al. 1997). It can be speculated that the entry of cytochrome c into the cytosol may not require a complete irreversible reduction in ΔΨₘ, but may be related to a transitory opening of the PTP, operating in a low conductance mode.

Like 90% of mitochondrial molecules, cytochrome c is encoded by a nuclear gene, translated as an extramitochondrial precursor, apocytochrome c, and imported into the mitochondrial inter-membrane space. The uptake of apocytochrome c follows a unique import pathway into the mitochondria, in that it does not require an import receptor, proteolytic processing of a precursor sequence, high ΔΨₘ, or ATP (Dumont et al., 1987; Nicholson et al., 1987; Drygas et al., 1989; Stuart and Neupert, 1990). Covalent attachment of a heme group catalyzed by the cytochrome c heme lyase promotes the import of apocytochrome c and leads to its final protein folding which has been proposed to capture cytochrome c within the mitochondria. Whether the unique import pathway of cytochrome c is related to its release from mitochondria of pre-apoptotic cells remains to be investigated.

Bcl-2 and Bcl-x₅ are located primarily in the outer mitochondrial membrane and prevent cytochrome c release from mitochondria, caspase activation and apoptosis (Yang and Korsmeyer, 1996; Kim et al., 1997; Kluck et al., 1997a; Kroemer, 1997b; Reed, 1997; Yang et al., 1997). This finding was confirmed here by confocal microscopy and immunolocalization of cytochrome c in HeLa cells with overexpression of Bcl-2 (Figure 7). The three-dimensional structure of Bcl-x₅ reveals structural similarities to pore-forming domains of some bacterial toxins (Muchmore et al., 1996). In addition, recombinant Bcl-x₅ or Bcl-2 form pH-sensitive, ion-conducting channels in planar lipid bilayers, suggesting that they may regulate cell survival by regulating membrane permeability (Minn et al., 1997; Schendel et al., 1997). Similarly, Bax, a pro-apoptotic member of the Bcl-2 family, forms a pH-sensitive, voltage-dependent ion-conducting channel in artificial lipid bilayers in vitro, which can be inhibited by Bcl-2 (Antonsson et al., 1997). Thus Bcl-2 family members may regulate the flow of ions across mitochondrial membranes and thereby provoke the release of cytochrome c from mitochondria. Furthermore, the channel properties of Bcl-2 might be regulated by associated molecules, for instance the Raf-1 kinase and the CED-4 homologs (H.-G. Wang et al., 1996; Chinnaiyan et al., 1997; James et al., 1997; Kroemer, 1997b; Reed, 1997; Spector et al., 1997; Wu et al., 1997). Although Bcl-x₅, Bcl-2 and Bax have been shown to function as ion-conducting channels in planar lipid bilayers, it cannot be excluded that they also act as protein channels under physiological conditions, regulating the passage of molecules such as cytochrome c. Alternatively, other mitochondrial molecules with channel properties may regulate the mitochondrial efflux of cytochrome c in apoptosis.

Consequences of mitochondrial loss of cytochrome c

In mitochondria, cytochrome c is required as an electron carrier in oxidative phosphorylation, a process which generates the majority of intracellular ATP. Cytochrome c accepts electrons from complex III and transfers them to complex IV (Hafeti, 1985; Mathews, 1985). The electron transport between these complexes generates a proton gradient across the inner mitochondrial membrane, which maintains ΔΨₘ. Electrons accepted from cytochrome c oxidase are then transferred to oxygen, which is reduced to water. Thus a long-term exclusion of cytochrome c from the electron transport chain can result in an impairment of proton flow and a decrease in ΔΨₘ generation of reactive oxygen species (ROS) due to incomplete reduction of oxygen, and a cessation in ATP synthesis (Hockenbery et al., 1991; Jacobson, 1996; Simonian and Coyle, 1996; Nicotera and Leist, 1997; Tsujimoto, 1997). Results in this report indicate, however, that at the time when cytochrome c enters the cytosol, ΔΨₘ and the intracellular ATP level are maintained for several hours after apoptosis induction by staurosporine (Figures 1 and 4). Several explanations may account for no immediate decrease in ΔΨₘ and ATP levels after the release of cytochrome c early in apoptosis. First, not all mitochondrial cytochrome c may actively participate in the electron transport chain, i.e. the released cytochrome c might represent an excess not involved in electron transport. Second, maintenance of ΔΨₘ may be accomplished by the import of cytosolic ATP through the ADP/ATP-translocator, generating a ΔΨₘ by the oligomycin-sensitive proton pump. Support for such a compensatory mechanism is provided by the observation that ρ₀ cells, which lack a functional electron transport chain due to deletion of their mitochondrial genome, exhibit a normal ΔΨₘ and are able to undergo PT and apoptosis (Jacobson et al., 1993; Marchetti et al., 1996a,b). Thus loss of mitochondrial cytochrome c does not necessitate a rapid collapse in ΔΨₘ. Similarly, our data show that intracellular ATP levels are maintained for several hours after apoptosis induction by staurosporine. A decrease in mitochondrial ATP synthesis may be
compensated for by an increase in anaerobic glycolysis. It is of note that cells in this study were cultured in medium containing a high level of glucose. In fact, it has been proposed that a complete de-energization of cells due to extensive mitochondrial damage and inhibition of glycolysis would preclude apoptosis. Several reports have shown that early events in apoptosis are comprised of several ATP-dependent steps, and the availability of intracellular ATP determines whether cells undergo apoptosis or necrosis (Kass et al., 1996; Richter et al., 1996; Eguchi et al., 1997; Leist et al., 1997; Nicotera and Leist, 1997; Tsujimoto, 1997). A depletion of the intracellular ATP pool in late apoptosis might be due to early ATP-consuming reactions, together with a complete loss of mitochondrial function later in the process.

**Molecular ordering of the cell death pathway**

A schematic representation of an apoptosis model is shown in Figure 8. Diverse cell death-transducing signals act upstream of mitochondria to induce, by an unknown mechanism, the mitochondrial release of cytochrome c. The cytochrome c efflux from mitochondria to cytosol can be prevented by the cell death antagonists, Bcl-2 or Bcl-xL, and may at least in part account for their cell survival properties (Golstein, 1997; Kim et al., 1997; Kluck et al., 1997a; Yang et al., 1997). Upon entering the cytosol, cytochrome c cooperates with at least two other factors (X), one of which is the recently identified Apaf-1 protein, in the proteolytic activation of the DEVD-specific caspsases (Liu et al., 1996; Kluck et al., 1997a,b; Vaux, 1997; Yang et al., 1997; Zou et al., 1997; S.J.Martin, R.M.Kluck, C.A.Casiano, D.D.Newmeyer, H.-G.Wang, J.C.Reed, D.D.Nicholson, E.S.Alnemri and D.R.Green, in preparation). Active caspsases cleave specific substrates, leading either to activation or inactivation of their target molecules and apoptotic changes. Caspase activity and cell death are prevented by zVAD-fmk. Caspsases also act upon mitochondria to induce PT, a reduction in $\Delta\Psi_{m}$, which triggers the liberation of AIF from mitochondria, and generation of ROS (Susin et al., 1996, 1997; Zamzami et al., 1996a,b; Boise and Thompson, 1997). AIF alone can provoke nuclear apoptosis. Bcl-2 and Bcl-xL block apoptosis by preventing cytochrome c release, downstream caspase activation, reduction in $\Delta\Psi_{m}$ and AIF liberation (Hennet et al., 1993; Zamzami et al., 1995a,b; Armstrong et al., 1996; Chinnaiyan et al., 1996; Shimizu et al., 1996a,b; Susin et al., 1996, 1997; Boise and Thompson, 1997; Kim et al., 1997; Kluck et al., 1997a; Yang et al., 1997).

Mitochondria and the entry of cytochrome c from mitochondria into the cytosol may play a central role in the regulation and activation of the executioner phase of apoptosis. Future investigations will focus on the identification of the upstream signals promoting the mitochondrial loss of cytochrome c in the early phase of apoptosis and the mechanisms whereby cytochrome c acts to activate DEVD-specific caspsases.

**Materials and methods**

**Reagents**

Anti-cytochrome c antibodies (TH8,2C12) were a gift from Ronald Jemmerison. Anti-caspase-3 (CPP32) antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-actin antibody was from ICN Chemical Credential (clone 14) (Aurora, OH); anti-PARP antibodies were from Pharmingen (San Diego, CA) (clone C2-10). Anti-human cytochrome c oxidase (subunit II) antibodies and the Mitotracker orange reagent CMTMRos were purchased from Molecular Probes (Eugene, OR). High fluorescent FITC-labeled goat anti-mouse antibodies were from Antibodies Incorporated (Davies, CA). Staurosporine, propidium iodide and CsA were from Sigma (St. Louis, MO). A cocktail of protease inhibitors (complete cocktail) was purchased from Boehringer Mannheim (Indianapolis, IN). Z-Val-Ala-Asp-CH2F (zVAD-fmk) was obtained from Kamiya Biomedical Company (Seattle, WA). DEVD-pNA was from Biomol Laboratories (Plymouth, PA). The TUNEL reagents were purchased from Oncor (Gaithersburg, MD). The SuperSignal Substrate was from Pierce (Rockford, IL).

**Cell culture**

CEM cells were cultured in RPMI 1460 medium (Gibco) supplemented with penicillin, streptomycin (50 U/ml), glutamate (2 mM) and 10% fetal bovine serum (FBS). HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) ( Irving Scientific) containing 4.5 g glucose/l and supplemented with penicillin, streptomycin and 10% FBS.

**Assessment of apoptosis**

Apoptotic cell death was measured by staining with annexin-V–FITC by a modified method described in Martin et al. (1995). A total of $5\times10^{6}$ cells were collected by centrifugation at 200 g (5 min) and cell pellets were resuspended in 100 $\mu$l of buffer containing 10 mM HEPES-KOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 1 mg/ml annexin-V–FITC (Bioproducts). After 15 min of incubation at
37°C, cells were spun onto glass slides (300 g, 5 min) and air dried for 5 min. Cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4 for 10 min and subsequently washed (three times, 5 min) with PBS, pH 7.4. The slides were then mounted with FluoroGuard Antifade Reagent (Bio-Rad) and apoptosis was assessed using a fluorescence microscope by counting FITC-positive cells. When apoptosis was quantified for HeLa cells, cells were stained with eosin plus hematoxylin stain, and apoptotic cells were counted using a light microscope. Apoptosis was detected by cell shrinkage, chromatin condensation and nuclear fragmentation.

**Immunocytochemistry and confocal microscopy**

HeLa cells were grown on 1 mm (Goldschmidt No.1) glass coverslips for 24–48 h in DMEM containing 10% FBS. When caspase inhibitors were used, cells were pre-treated for 1 h with 100 μM zVAD-fmk peptide inhibitor. Apoptosis was induced by UVB irradiation (14 J/cm²). Four hours later, the cells were rinsed once with PBS and then fixed (2 min) with methanol/aceton (1:1) at room temperature. After fixation, glass coverslips were air dried and incubated with anti-cytochrome c antibodies (7H2,2C12) diluted 1:150 in PBS, 3% bovine serum albumin (BSA) for 2–3 h at 37°C in a humidified chamber. Excess antibody binding was removed by washing the coverslips with PBS (three times, 5 min). The secondary antibody, a high fluorescence FITC-labeled goat anti-mouse antibody (Antibodies Incorporated), diluted 1:10 in PBS, 3% BSA, was added and incubated for 1 h at room temperature, with protection against light. Coverslips were then rinsed with PBS for 3 min, incubated for 1 min with PBS containing 500 ng/ml of propidium iodide (Sigma) and washed with PBS (three times, 5 min). Finally, the coverslips were mounted onto microscope slides using FluoroGuard Antifade Reagent (Bio-Rad) and analyzed using a Zeiss LMS 410 inverted laser scanning microscope equipped with a 488 nm argon laser when FITC labeling was analyzed and a 543 nm helium neon laser when propidium iodide staining was assessed.

**Confocal microscopy analysis of mitochondrial transmembrane potential and DNA fragmentation**

HeLa cells were seeded at low density on 1 mm coverslips and cultured for 48 h in DMEM containing 10% FBS. Apoptosis was induced either by UVB light (14 J/cm²) or staurosporine (1 μM). Cells were loaded with the CMTMRos dye (150 nM) in culture medium for 15–45 min, at 37°C and 5% CO₂. Cells were then washed once with PBS and fixed with 4% paraformaldehyde in PBS, pH 7.4 for 10 min. Coverslips were then washed with PBS, pH 7.4 (three times, 5 min) and then analyzed for DNA fragmentation using the TUNEL fluorescence labeling kit from Oncor, following the instructions provided by the manufacturer. Finally, the slides were mounted onto glass microscope slides using the FluoroGuard Antifade Reagent (Bio-Rad) and analyzed by confocal microscopy as described above.

**Preparation of cytosolic extracts and immunoblotting**

CEM cells were collected by centrifugation at 200 g for 5 min and at 4°C. The cells were then washed twice with ice-cold PBS, pH 7.4, followed by centrifugation at 200 g for 5 min. The cell pellets were resuspended in 600 μl of extraction buffer, containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM dithiothreitol (DTT) and protease inhibitors (Complete Cocktail, Boehringer Mannheim). After 30 min incubation on ice, cells were homogenized with a glass dounce and a B pestle (40 strokes). Cell homogenates were spun at 14 000 g for 15 min and supernatants were removed and stored at −70°C, until analysis by gel electrophoresis. Fifty μg of cytosolic protein extracts were loaded onto each lane of an 8 or 15% SDS–polyacrylamide gel and separated and then blotted to PVDF transfer membrane (0.2 μm) (Bio-Rad), when cytochrome c or pro-caspase-3 protein was analyzed or to Hybond-ECL nitrocellulose membrane (0.45 μm) (Amersham) when PARP and cytochrome c oxidase (subunit II) or actin were analyzed, at 150 mA overnight in transfer buffer (20 mM Tris-base, 150 mM glycine, 20% methanol). Non-specific binding was blocked by incubation in 3% BSA, 3% non-fat milk, 0.1% Tween-20, in PBS for 3 h at room temperature. Anti-cytochrome c monoclonal antibody 7H2,2C12 was diluted 1:2000; pro-caspase-3-specific antibody was diluted 1:250; PARP-specific antibodies were diluted 1:1000; actin-specific antibodies were diluted 1:3000; cytochrome oxidase (subunit II) antibodies were diluted 1:300 in PBS, containing 3% non-fat milk, 0.05% Tween-20. After overnight incubation with agitation, at 4°C, filters were washed three times with PBS, 3% non-fat milk and 0.1% Tween-20. The secondary antibody, a horseradish peroxidase-coupled donkey anti-mouse antibody (Amersham), was incu-

**Measurement of ΔΨm by flow cytometry**

To measure ΔΨm, the mitotracker orange reagent CMTMRos was used as described by Macho et al. (1996). Briefly, 5×10⁶ cells were incubated with CMTMRos (150 nM) for 15–30 min in culture medium, at 37°C and 5% CO₂. As a positive control for ΔΨm loss, CEM cells were incubated at the same time with uncoupling agent MCI CCCP (50 μM), a protonophore, which disrupts ΔΨm. The cells were washed once with PBS, collected by centrifugation at 200 g and fixed in 2 ml of 4% paraformaldehyde in PBS pH 7.4 for 15 min at room temperature using a horizontal shaker (150 rpm). Fixed cells were then stored in the dark for 1–7 days at 4°C prior to analysis by flow cytometry (Becton/Dickinson) using excitation of a single 488 nm argon laser. Data acquisition and analysis were performed using the CellQuest software. Forward scatter, perpendicular scatter and CMTMRos fluorescence were measured with a 585/42 nm band pass filter. At least 10 000 events were collected per sample, with the CMTMRos fluorescence recorded as logarithmic amplified data.

**Measurement of DEVD-pNA cleavage activity**

Cytosolic extracts were prepared as described above and stored at −70°C. Proteolytic reactions were carried out in extraction buffer, containing 20 μg of cytosolic protein extracts and 40 μM N-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA) (BIOMOL Research Laboratories). The reaction mixtures were incubated at room temperature for 2 h, and the formation of p-nitroanilide was measured at 405 nm using a Spectra Max 250 microtiter plate reader.

**Measurement of ATP level**

A total of 10⁵ cells were washed twice with ice-cold PBS, pH 7.4, collected by centrifugation at 200 g, resuspended in 200 μl of boiling 100 mM Tris-HCl pH 7.75, 4 mM EDTA and incubated for another 10 min at 100°C. Samples were then centrifuged at 10 000 g for 1 min and the supernatants were kept on ice or stored at −70°C until ATP measurement. ATP levels were measured with the Boehringer Mannheim ATP Bioluminescence Assay Kit CLS II. Fifty μl of sample was mixed gently with 50 μl of luciferase reagent in borsilicated glass tubes, and luciferase activity was measured for 10 s using a Monolight 2010 analytical luminescence reader.

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**References**

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