

Disruption of Mitochondrial Function during Apoptosis Is Mediated by Caspase Cleavage of the p75 Subunit of Complex I of the Electron Transport Chain

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Summary

Mitochondrial outer membrane permeabilization and cytochrome c release promote caspase activation and execution of apoptosis through cleavage of specific caspase substrates in the cell. Among the first targets of activated caspases are the permeabilized mitochondria themselves, leading to disruption of electron transport, loss of mitochondrial transmembrane potential ($\Delta\Psi_m$), decline in ATP levels, production of reactive oxygen species (ROS), and loss of mitochondrial structural integrity. Here, we identify NDUF51, the 75 kDa subunit of respiratory complex I, as a critical caspase substrate in the mitochondria. Cells expressing a noncleavable mutant of p75 sustain $\Delta\Psi_m$ and ATP levels during apoptosis, and ROS production in response to apoptotic stimuli is dampened. While cytochrome c release and DNA fragmentation are unaffected by the noncleavable p75 mutant, mitochondrial morphology of dying cells is maintained, and loss of plasma membrane integrity is delayed. Therefore, caspase cleavage of NDUF51 is required for several mitochondrial changes associated with apoptosis.

Introduction

The process of apoptosis is orchestrated by the activation of “executioner” caspases (caspase-3 and caspase-7 in mammals) and subsequent cleavage of their substrates (Earnshaw et al., 1999; Green and Evan, 2002). In vertebrates, the best-characterized mechanisms for caspase activation during apoptosis are those of the death receptor and mitochondrial pathways (Green and Evan, 2002), and the latter accounts for most forms of apoptosis in response to cellular stress, loss of survival factors, and developmental cues. The mito-

chondrial pathway is engaged when members of the BH3-only subset of the Bcl-2 family proteins (e.g., Bid and Bim) activate another subset, the multidomain/BH123 proteins (e.g., Bax and Bak). The latter then permeabilize the mitochondrial outer membrane. This leads to the release of cytochrome c and other proteins contained in the mitochondrial intermembrane space (Newmeyer and Ferguson-Miller, 2003; Ricci et al., 2003b). Cytochrome c induces the oligomerization of cytosolic APAF-1, which in turn recruits and activates the cytosolic initiator caspase caspase-9. The resulting apoptosome complex contains active caspase-9, which cleaves and activates the executioner caspases caspase-3 and caspase-7 (Li et al., 1997). It is these executioner caspases, especially caspase-3, that cleave specific substrates within the cell to produce the changes associated with apoptosis.

The OXPHOS system consists of four multimeric complexes (in addition of coenzyme Q and cytochrome c) forming the mitochondrial respiratory chain (complexes I to IV). This chain transfers electrons from the reducing equivalent (NADH-FADH₂) to molecular oxygen, creating a proton gradient across the inner mitochondrial membrane, measured as $\Delta\Psi_m$. This force is used by complex V (F₀F₁ ATPase) to produce ATP. $\Delta\Psi_m$ is not only important for ATP production but is also required for mitochondrial protein import and to regulate metabolite transport. Therefore, $\Delta\Psi_m$ is often employed as an indicator of cellular viability, and its disruption has been implicated in a variety of apoptotic phenomena (Marchetti et al., 1996; Zamzami et al., 1995a, 1995b). $\Delta\Psi_m$ loss during apoptosis was widely taken as evidence for the involvement of a permeability transition in the process, in which the adenine nucleotide translocator in the inner membrane opens to dispel the gradient (Bauer et al., 1999), although recent evidence suggests that the mitochondrial permeability transition (MPT) can proceed in the absence of an adenosine nucleotide transporter (ANT) (Kokoszka et al., 2004).

Studies in single cells, however, established that the loss of $\Delta\Psi_m$ during apoptosis generally follows cytochrome c release (De Giorgi et al., 2002; Goldstein et al., 2000; Waterhouse et al., 2001). Further, inhibition of caspase activation or use of cells lacking APAF-1 revealed that the rapid loss of $\Delta\Psi_m$ following cytochrome c release depends on caspase activity (Bossy-Wetzel et al., 1998; Goldstein et al., 2000; Waterhouse et al., 2001). Remarkably, in the absence of caspase activation, the low levels of cytochrome c dispersed throughout the cell can be sufficient to maintain function of the electron transport chain and ATP synthesis in the permeabilized mitochondria (Waterhouse et al., 2001). This has raised the possibility that the MPT is not required for mitochondrial outer membrane permeabilization (MOMP) or cytochrome c release. Thus, while in some settings MPT may participate in apoptosis, in many it may not.

More recently, the site of action of caspases in the electron transport chain was localized to complexes I and II, while complexes III and IV were found to remain

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functional after exposure of isolated, permeabilized mitochondria to caspases and in apoptotic cells (Ricci et al., 2003a). The results of this assault by caspases on mitochondria are the loss of $\Delta\Psi_m$ and the production of reactive oxygen species (ROS). The role of this effect in the orchestration of cell death has not been determined.

Caspases orchestrate the process of apoptosis through cleavage of specific substrates in the cell. While many other caspase substrates are known, most have no defined function during apoptosis (reviewed in Fischer et al. [2003]). The substrates ICAD (Enari et al., 1998) and Acinus (Sahara et al., 1999) are responsible for DNA fragmentation and nuclear condensation, respectively, while p21-activated kinase (Rudel et al., 1998), gelsolin (Kothakota et al., 1997), and ROCK-1 (Coleman et al., 2001) mediate blebbing following caspase activation. None of these account for the mitochondrial changes observed during apoptosis.

In this study, we employed a novel approach to identify the 75 kDa subunit of complex I as a caspase substrate accessible to the intermembrane space of the mitochondria. Mutation of the single, conserved caspase cleavage site leads to protection of mitochondrial functions and integrity during apoptosis. This mitochondrial protection was coupled with a delay in plasma membrane disruption during apoptosis. Caspase-induced changes in mitochondrial functions are therefore likely to have a central role in caspase-dependent cell death.

Results

To identify potential caspase substrates in mitochondria, we developed the strategy outlined in Figure 1A. After isolation from mouse liver, soluble and membrane-associated proteins were further purified from mitochondria and then resolved by SDS-PAGE (first dimension, see Experimental Procedures for details). The lane was excised, dehydrated, and then rehydrated in the presence of recombinant active caspase-3, allowing the caspase access to substrates in the gel. Proteins were then further resolved in the second dimension by SDS-PAGE. While the majority of proteins are expected to appear on the diagonal (corresponding to intact proteins), caspase substrates should be cleaved and therefore resolve as distinct spots below the diagonal. This technique was validated using whole cell lysates, and the known caspase-3 substrate poly-ADP ribose polymerase (PARP) was identified by Western blot. As shown in Figure 1B, PARP appeared as a single spot located on the gel's diagonal when no caspase was used. Treatment of the first dimension with caspase-3 revealed the cleaved (p85) fragment of PARP under the diagonal.

Mitochondrial proteins were subjected to this simple procedure (Figure 1C), and discrete spots were analyzed by MALDI-TOF. Two of these, found aligned in the second dimension, were identified as two fragments of the 75 kDa subunit of complex I (also called NADH dehydrogenase Fe-S protein-1, NDUFS1, or NUAM). The residues at the junction of these fragments conform to a consensus caspase-3/7 site (Stennicke et al., 2000), DxxD/A, and are highly conserved in animals (Figure 1D).

Complex I is a large multienzyme complex composed

of at least 46 subunits with a combined molecular mass of about 1000 kDa (Walker, 1992). Of the 46 subunits, seven are encoded in the mitochondrial genome, while the others are nuclear encoded and imported after translation (Robinson, 1998). Complex I has been identified as a major source of ROS (Li et al., 2003) and is the major route of entry of electrons into the respiratory chain. We therefore sought to determine how caspase cleavage of p75 impacts on mitochondrial function and what affects this has in the process of apoptosis.

To confirm that p75 can be a substrate for caspases, human p75 was cloned with a V5 tag added to the C terminus and expressed by *in vitro* transcription and translation. Treatment of the protein with recombinant caspase-3 produced the expected 47 and 28 kDa fragments, plus some others (Figure 2A). However, mutation of the presumptive target site, converting Asp 255 to Ala, rendered the protein resistant to caspase-3 (Figure 2A), confirming that there is a single cleavage site.

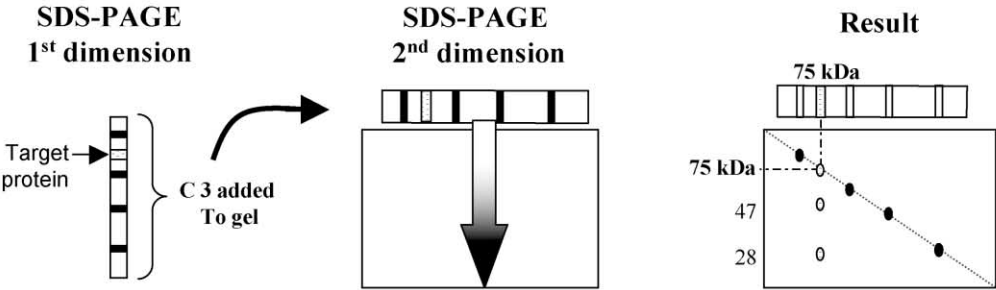
We therefore tested if p75 is cleaved by caspases during apoptosis. As shown in Figures 2Ba, 2Bb, and 2Bc, immunoblots showed a loss of p75 during apoptosis induced by staurosporine, UV, or TNF/CHX, which was blocked by addition of the caspase inhibitor QVD-fmk. Another subunit of complex I, which was not cleaved during cell death (p17, see also Figure 2C), served as a loading control. Several other components of complex I remained intact upon induction of apoptosis (Figure 2C). As expected, the nuclear caspase substrate PARP was cleaved in the apoptotic cells.

The accessibility of the p75 subunit to the intermembrane space (IMS) is controversial. Two early studies demonstrated that this subunit can be accessed from the IMS (Ohnishi et al., 1985; Smith and Ragan, 1980). More recently, complex I organization has been resolved into various subcomplexes named I α , β , and λ (Carroll et al. [2003], for review, see Hirst et al. [2003]), and it has been suggested that p75 is exclusively contained within the I λ subcomplex, located in the matrix. However, in that study, p75 was found not only in fraction I λ (located in the matrix) but to the same extent in fraction I α (potentially accessible to the IMS) (Carroll et al., 2003).

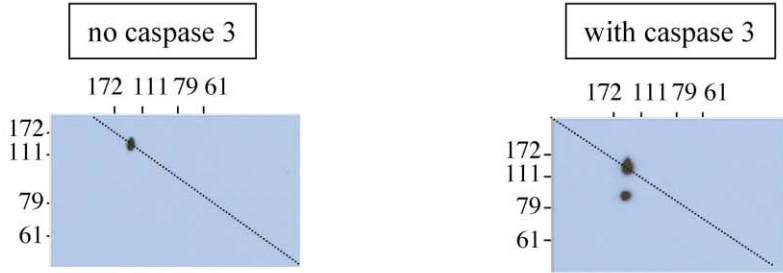
Therefore, in order to examine the accessibility of p75 to the intermembrane space, we prepared mitochondria and induced MOMP using ^{35}S -MOMP, as described (von Ahn et al., 2000). Permeabilization was assessed by cytochrome c release (Figure 2Db). The permeabilized mitochondria were then treated with trypsin. As shown in Figure 2Da, trypsin effectively digested p75 but not mtHSP60 (which is trypsin sensitive when treated directly, data not shown) in the permeabilized mitochondria. Since mtHSP60, which is localized in the mitochondrial matrix, was not digested, the digestion of p75 indicates that this protein is accessible to the intermembrane space. Further confirmation of the localization of p75 was obtained using mitoplasts from cells ectopically expressing tagged p75 (see below and Supplemental Figure S1E at <http://www.cell.com/cgi/content/full/117/6/773/DC1>).

During apoptosis, the mitochondrial outer membrane becomes permeable (and this is necessary for caspase activation in the mitochondrial pathway [Newmeyer and Ferguson-Miller, 2003]), and activated caspases in the

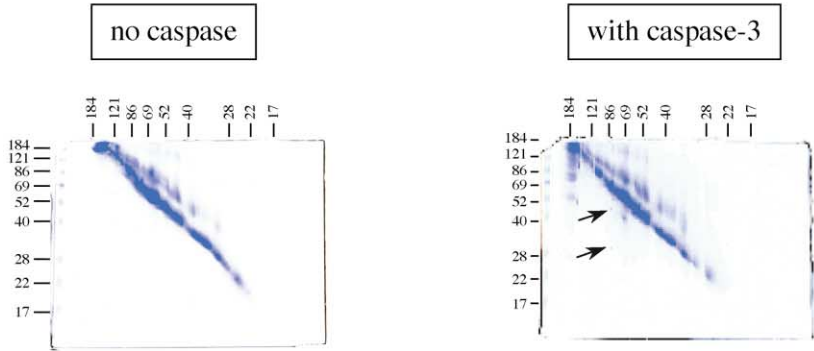
A



B



C



D

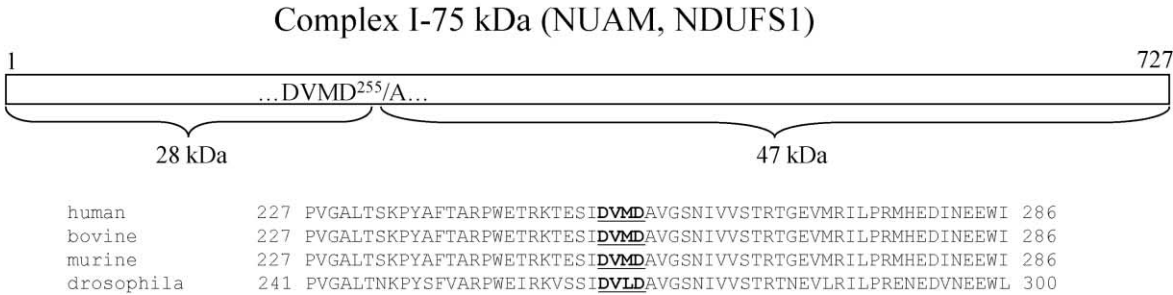


Figure 1. Identification of p75 as a Caspase Substrate by Diagonal Gel

(A) Diagonal gel technique for identification of potential caspase substrates. See Experimental Procedures for details.

(B) The caspase substrate PARP is revealed by diagonal gel. Whole extracts from HeLa cells were resolved by SDS-PAGE, and the lane was rehydrated in the presence or absence of recombinant caspase-3 and again resolved by SDS-PAGE in the second dimension. PARP was visualized by Western blot. The dotted line represents the diagonal.

(C) Coomassie-stained diagonal gels of membrane-associated mitochondrial proteins with (right) or without (left) caspase-3. The two arrows indicate the spots corresponding to the 47 and 28 kDa subunits of p75 as determined by MALDI-TOF.

(D) The caspase cleavage site of p75 (NUAM/NDUFS1) and alignment of the region containing the caspase cleavage site of p75 from several species.

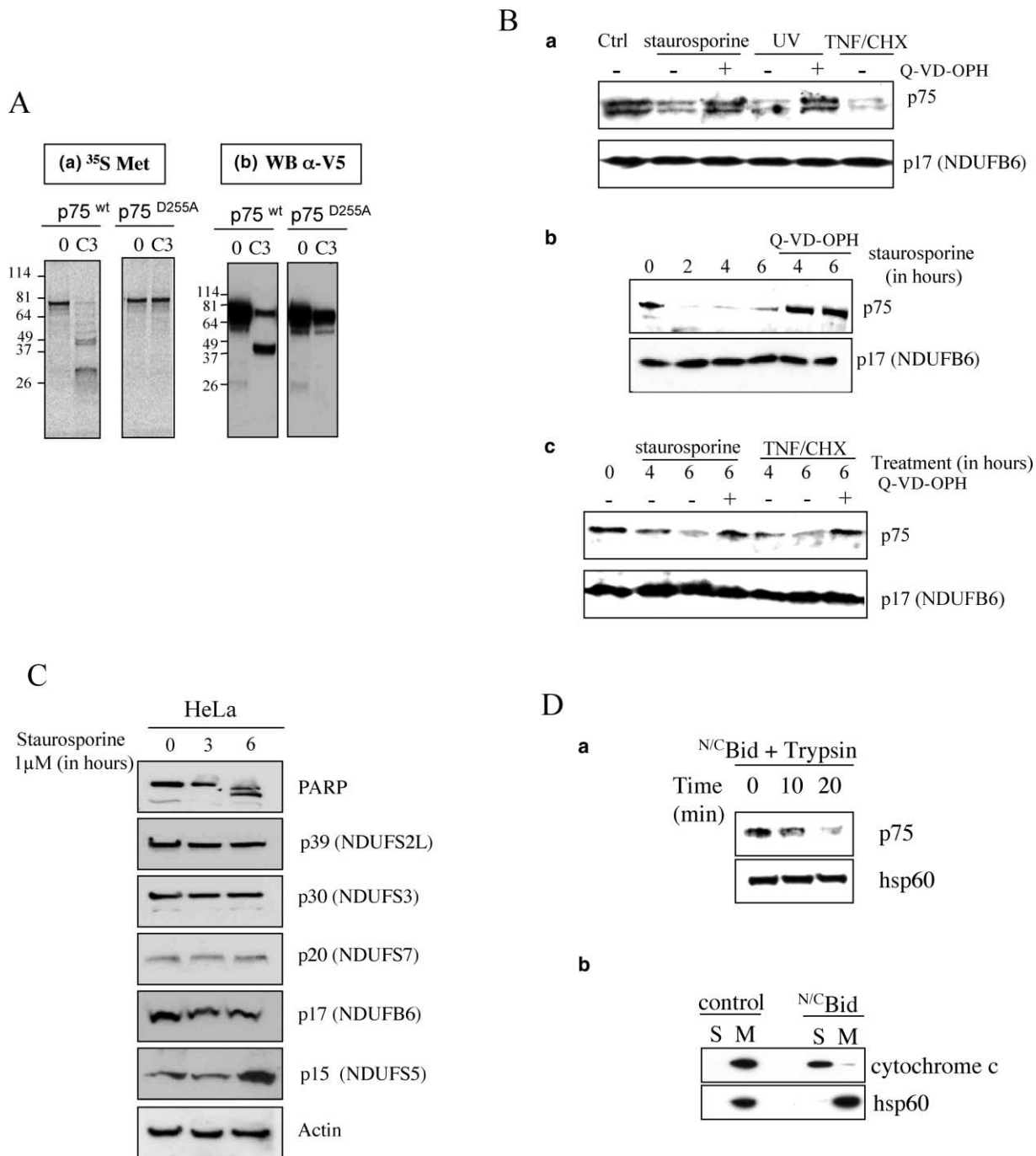


Figure 2. p75 Is a Caspase Substrate

(A) (Aa) Human p75^{wt} and p75^{D255A} were expressed by in vitro transcription and translation in the presence of ³⁵S Met, then incubated with or without recombinant caspase-3 (10 μg/ml) for 30 min at 37°C. (Ab) C-terminal V5-tagged constructs were expressed, and the extracts were subjected to Western blot using a C-terminal anti-V5 tag antibody.

(B) Cleavage of p75 by caspases during apoptosis. (Ba) HeLa cells were treated for 6 hr with staurosporine (1 μM), UV (600 mJ/cm²), or TNF/CHX (100 ng/ml and 10 μM, respectively) in the presence or absence of Q-VD-OPH (20 μM). Mitochondria were isolated and proteins resolved by SDS-PAGE. The two bands correspond to the expected sizes of the proform (with mitochondrial import sequence) and processed forms of p75 (the proform was not observed in most experiments). (Bb and Bc) HeLa cells were treated with 1 μM staurosporine or TNF/CHX (100 ng/ml and 10 μM, respectively) for the indicated times with or without the caspase inhibitor Q-VD-OPH (20 μM) and then analyzed as in (Ba). Endogenous p75 and the complex I subunit p17 (NDUFB6) were assessed by Western blot.

(C) Other complex I subunits are not cleaved during apoptosis. HeLa cells were treated with staurosporine for the indicated times, and several subunits of complex-I were visualized by Western blot during apoptosis.

(D) (Da) Mitochondria from HeLa cells were incubated in the presence of ^{N/C}Bid (20 μg/ml) for 1 hr at 37°C, and then trypsin (10 μg/ml) was added at 37°C for the indicated time. Degradation of the endogenous p75 but not hsp60 was assessed by Western blot. (Db) Permeabilization of the OMM but not the IMM was examined in the samples in (Da) by assessing the release of cytochrome c versus hsp60 from the mitochondria (M) into the supernatant (S).

cytosol then could gain access to proteins exposed to the intermembrane space. The caspase cleavage site of p75 is therefore most likely accessible to caspases that have entered the permeable mitochondria.

Stable lines expressing p75^{wt} or p75^{D255A} were generated in HeLa. In these lines, p75-V5 was localized exclusively to the mitochondrial pellet (Figure 3Aa). By immunostaining for the V5 tag, cells expressed similar levels of either form of p75-V5 and showed no differences in steady-state levels of $\Delta\Psi_m$ or superoxide production (see Supplemental Figures S1A–S1C at Cell website). Immunoblot with anti-p75 antibody showed that the level of expression of either form of p75 increased total p75 levels only slightly, indicating that these were not dramatically overexpressed (Figure 3Ab). Both forms of p75-V5 (wild-type [wt] and D²⁵⁵A) were coimmunoprecipitated with p20 (NDUFS7), another subunit of complex I (Supplemental Figure S1D), indicating that at least some of the tagged forms of p75 are incorporated in the multimolecular complex I. Both forms of the tagged p75 were digested by trypsin treatment of mitoplasts but not mitochondria under conditions where mtHSP60 was protected (Supplemental Figure S1E).

To further examine the function of the two ectopically expressed forms of p75, we employed siRNA to knock down the levels of endogenous p75. Treatment of HeLa with siRNA for p75 greatly reduced levels of endogenous p75 but did not affect expression of p75^{wt} or p75^{D255A} (Supplemental Figure S2A). Cells were then digitonin permeabilized, and $\Delta\Psi_m$ was assessed using TMRE. Although the siRNA treatment had only a minimum effect on $\Delta\Psi_m$ (presumably because complex II function remained intact), upon addition of the complex I substrates malate and glutamate, $\Delta\Psi_m$ increased dramatically. In contrast, HeLa cells treated with p75 siRNA did not show this complex I response (Supplemental Figure S2B). Cells expressing p75^{wt} or p75^{D255A}, however, continued to display this increase in $\Delta\Psi_m$ in response to malate plus glutamate, suggesting that complex I activity was sustained in these cells. Therefore, both forms of p75 are likely to function in complex I.

If caspase-mediated disruption of p75 plays a role in apoptosis, then any effect of expression of the noncleavable p75 mutant would be expected to be downstream of cytochrome c release since, in the mitochondrial pathway, this occurs prior to caspase activation (Li et al., 1997). We therefore introduced cytochrome c-GFP into the HeLa lines expressing p75^{wt} or p75^{D255A} and analyzed cytochrome c release as described (Goldstein et al., 2000). No differences in the kinetics or extent of cytochrome c release were observed in apoptosis induced with either staurosporine or actinomycin D (Figure 3B).

As with native p75, the ectopically expressed p75^{wt} was cleaved during apoptosis, and the expected cleavage product was observed (Figure 3C). This cleavage product was found at lower levels than the intact p75, probably due to degradation as apoptosis progressed and mitochondria were disrupted (see below). In contrast, p75^{D255A} was not cleaved during apoptosis.

We then examined the effects of expression of wt or noncleavable p75 on mitochondrial respiration during apoptosis. HeLa cells expressing either form of p75 were permeabilized with digitonin, and respiration in response to different substrates was assessed by oxygen

consumption (Figures 3Da and 3Db). The responses of these cells to complex I and complex II substrates were similar. To determine the effects of apoptosis on mitochondrial respiration, cells were treated with staurosporine and then analyzed in the same way. Cells expressing p75^{wt} failed to respire in response to complex I or complex II substrates (Figure 3Dc), as described (Ricci et al., 2003a). In contrast, cells expressing p75^{D255A} continued to respire in response to complex I substrates but not in response to complex II substrate (Figure 3Dd, quantitation in Figure 3E). Therefore, the expression of the noncleavable p75 protected complex I activity (but not complex II) during apoptosis.

Previously, we showed that addition of caspase-3 to digitonin-permeabilized cells causes mitochondrial outer membrane permeabilization (Waterhouse et al., 2001), loss of respiration, and loss of $\Delta\Psi_m$ in response to substrates for complex I or complex II (Ricci et al., 2003a). The effects of the stable expression of the two forms of p75 on $\Delta\Psi_m$ in digitonin-permeabilized HeLa cells treated with caspase-3 were therefore assessed. The D²⁵⁵A mutant of p75 effectively protected $\Delta\Psi_m$ (Supplemental Figure S3A). Under these conditions, wt but not mutant p75 was cleaved (Supplemental Figures S3B and S3C), and this required mitochondrial permeabilization (presumably induced by caspase-3 cleavage of Bid [Li et al., 1998]), as Bcl-xL blocked caspase cleavage of the wt p75 (Supplemental Figure S3C).

During apoptosis, HeLa cells expressing p75^{wt} displayed a pronounced drop in $\Delta\Psi_m$ that was substantially inhibited by addition of a caspase inhibitor, consistent with our earlier observations (Goldstein et al., 2000; Waterhouse et al., 2001) (Figure 4A). In contrast, $\Delta\Psi_m$ was sustained in the cells expressing p75^{D255A} (Figure 4Aa). This effect was unaffected by the addition of oligomycin (Figure 4Ab), indicating that the maintenance of $\Delta\Psi_m$ was not through reversal of complex V. In contrast, the complex I inhibitor rotenone (which did not dissipate $\Delta\Psi_m$ in viable cells) eliminated the protective effect of p75^{D255A} on $\Delta\Psi_m$ in apoptotic cells (Figure 4Ac). The effect of rotenone was most likely due to the combined effect of inhibiting complex I function with rotenone in the protected cells, while complex II function was disrupted by the action of caspases (Ricci et al., 2003a).

This effect of expression of p75^{D255A} on maintenance of $\Delta\Psi_m$ during apoptosis was also observed in H1299 cells (Supplemental Figure S4B). The protection by p75^{D255A} was slightly enhanced by siRNA treatment to decrease the effects of the endogenous p75 (Supplemental Figure S2C).

To further examine this effect of p75^{D255A} on $\Delta\Psi_m$ during apoptosis, we generated additional forms of the p75 protein (Figure 4Ad). These were the N-terminal fragment of p75 following caspase cleavage, the corresponding C-terminal fragment (with the mitochondrial import sequence of p75), and an inactivating mutant of p75 (R²⁴¹W) (Benit et al., 2001) that was introduced into p75^{wt} and p75^{D255A}. Each of these, as well as p75^{wt} and p75^{D255A}, were transduced into HeLa cells by retrovirus and the change in $\Delta\Psi_m$ during apoptosis examined. Of all of these, only p75^{D255A} protected $\Delta\Psi_m$ during apoptosis (Figure 4Ad). Therefore, it appears that p75^{D255A} must be functional to protect mitochondrial function during apoptosis, and, further, it is the loss of this function

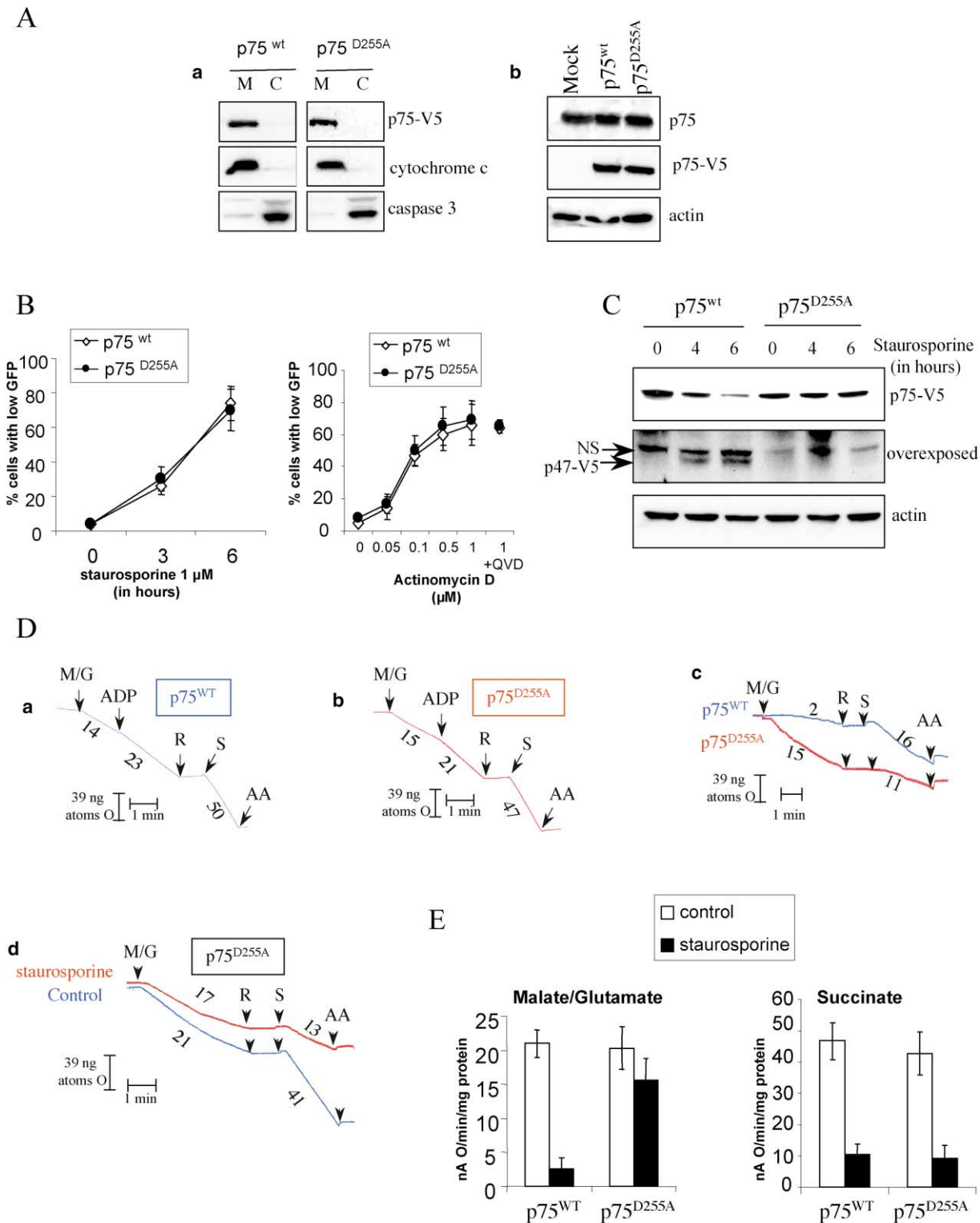


Figure 3. HeLa Cells Expressing p75^{D255A} but Not p75^{wt} Maintain Respiration during Apoptosis

(A) (Aa) HeLa cells stably expressing the wt or the D²⁵⁵A form of p75-V5 were fractionated into heavy membrane (M) and cytosolic (C) fractions. The presence of p75-V5 was determined by Western blot using an anti-V5 antibody. Cytochrome c and caspase-3 were used as fractionation controls. (Ab) Level of expression of the endogenous and V5-tagged forms of p75.

(B) Effect of p75 cleavage on cytochrome c release. HeLa cells expressing cytochrome c-GFP and the wt or the D²⁵⁵A form of p75-V5 were treated as indicated (Actinomycin D treatment, 18 hr). Where indicated, Q-VD-OPH (20 μ M) was added 10 min before treatment. Cytochrome c-GFP release was assessed by flow cytometry after cells were permeabilized with digitonin and measured as the loss of GFP fluorescence from the cells (Goldstein, et al., 2000).

upon caspase cleavage rather than another activity of the p75 fragments that is responsible for the effects of caspases on complex I activity.

The p75^{D255A}-expressing cells sustaining $\Delta\Psi_m$ during apoptosis had nevertheless released cytochrome c, as determined by simultaneous flow cytometry analysis of cytochrome c-GFP release and $\Delta\Psi_m$ and confocal imaging (Supplemental Figures S5A and S5B). We also performed time-lapse confocal microscopy in which HeLa cells expressing cytochrome c-GFP and the wt or the D²⁵⁵A mutant of p75-V5 were induced to undergo apoptosis (Figure 4B and Supplemental Movie S1). In cells expressing p75^{wt}, $\Delta\Psi_m$ was lost shortly after cytochrome c release. The cells then rounded and lost integrity. Cells expressing p75^{D255A} also showed a drop in $\Delta\Psi_m$ after cytochrome c-GFP release, but then $\Delta\Psi_m$ recovered for a significant period of time. Previously, we had observed such loss and recovery of $\Delta\Psi_m$ following cytochrome c release in cells induced to undergo apoptosis in the presence of caspase inhibitors (Waterhouse et al., 2001). In the experiment in Figure 4B, however, the cells that recovered $\Delta\Psi_m$ were nevertheless undergoing characteristic apoptotic death, presumably as a consequence of caspase activation.

Cells undergoing apoptosis produce ROS, and this was observed in the line expressing p75^{wt} to the same extent as in the control HeLa cells (Figure 4C). Again, production of ROS during apoptosis was significantly reduced in cells expressing p75^{D255A}.

Of course, the major function of the electron transport chain is the production of ATP, and, upon caspase activation, ATP levels rapidly fall in apoptotic cells (Waterhouse et al., 2001). This drop in ATP levels was observed in p75^{wt}-expressing cells undergoing apoptosis (Figure 4D), and the effect was inhibited by the addition of caspase inhibitors as described (Waterhouse et al., 2001). In contrast, ATP levels in the cells expressing p75^{D255A} were maintained during apoptosis (Figure 4D). Altogether, these results indicate that preventing p75 cleavage was sufficient for preserving most mitochondrial functions during apoptosis.

During apoptosis, mitochondria can undergo extensive morphological changes, including mitochondrial swelling and deterioration of matrix structure. While this was originally thought to reflect a mechanism of cytochrome c release (Marzo et al., 1998), subsequent studies showed that such changes follow cytochrome c release and can be caspase dependent (Dinsdale et al., 1999; Zhuang et al., 1998). Therefore, we examined the effect of caspase cleavage of p75 on mitochondrial morphology during apoptosis. HeLa cells were treated with staurosporine and examined at the ultrastructural level.

The morphology of mitochondria in untreated cells expressing p75^{wt} or p75^{D255A} was similar and normal in appearance (Figure 5A, Supplemental Figure S6). After 4 or 7 hr of treatment with staurosporine, the mitochondria in p75^{wt}-expressing cells frequently showed disrupted and swollen matrix and deformed structure (Figure 5B). In contrast, many mitochondria in treated cells expressing p75^{D255A} appeared normal. In each case, cells were compared that exhibited similar DNA condensation, indicating that they were undergoing apoptosis (Supplemental Figure S6). Overall, significant numbers of mitochondria of normal appearance were seen in apoptotic cells expressing p75^{D255A}, while these were only rarely seen in apoptotic cells expressing p75^{wt} (Figure 5B). Expression of p75^{D255A} therefore protected mitochondria from caspase-mediated disruption occurring during apoptosis.

Two major sites of action of executioner caspases are the chromatin and the plasma membrane. During apoptosis, caspases activate the caspase-activated DNase by cleaving its chaperone and inhibitor ICAD (Enari et al., 1998), resulting in DNA fragmentation. As shown in Figure 6A, this process was essentially identical in cells expressing p75^{wt} or p75^{D255A}. In the plasma membrane, caspases induce a redistribution of phosphatidylserine (PS) from the inner leaflet to both sides (PS "scrambling"), followed by a loss of plasma membrane integrity (Fadok et al., 2000; Martin et al., 1995). Despite identical DNA fragmentation kinetics, loss of plasma membrane integrity (uptake of PI) appeared to be delayed in the cells expressing p75^{D255A} versus those expressing p75^{wt} (Figure 6B). Expression of p75^{D255A} delayed the loss of plasma membrane integrity (measured by PI uptake) with no increase in cells with externalized PS (Annexin V⁺, PI⁻) (Figure 6B). If the effect were specific to loss of plasma membrane integrity, we might have expected an accumulation of the Annexin V⁺, PI⁻, which precedes this event. Therefore, expression of p75^{D255A} is likely to delay PS externalization as well as loss of plasma membrane integrity.

Using time lapse microscopy (Figure 6C, Supplemental Movie S2), we found that the interval between cytochrome c release and PS externalization was similar in the two cell lines, while the uptake of PI after cytochrome c release was significantly delayed (Figure 6C inset and Supplemental Figure S7). This effect was also observed in H1299 cells expressing p75^{D255A} (Supplemental Figure S4C). It is possible that this maintenance of plasma membrane integrity is a reflection of the sustained levels of ATP during apoptosis in the cells expressing p75^{D255A} (Figure 4D). The apparent failure to observe a delay in externalization of PS at the single cell level (Figure 6C

(C) Cells expressing the wt or D²⁵⁵A form of p75-V5 were incubated with staurosporine (1 μ M) for the indicated times. Only the wt form of p75 was cleaved during apoptosis. NS, nonspecific.

(D) Respiration during apoptosis. Values for oxygen consumption are represented on the curves as ng atoms of oxygen/min/mg of protein. (Da and Db) Respiration of HeLa cells expressing the wt or D²⁵⁵A forms of p75. After permeabilization, respiration in response to complex I (malate/glutamate: M/G, 5 mM each) or complex II (succinate: S, 5 mM) substrates was measured. Respiratory inhibitors were used in our studies as indicated: rotenone (R, 2 μ M) and antimycin A (AA, 1 μ M). (Dc) Respiration during apoptosis. Cells expressing the wt or mutant forms of p75 were incubated in the presence of staurosporine (1 μ M) for 6 hr to induce apoptosis, and oxygen consumption was assessed as in (Da) and (Db). (Dd) Respiration during apoptosis. As in (Dc), but comparing D²⁵⁵A cells with or without staurosporine treatment to induce apoptosis. In (Dc) and (Dd), ADP (2 mM) and cytochrome c (100 μ M) were added to the respiration buffer prior to measurement.

(E) Means of three independent experiments.

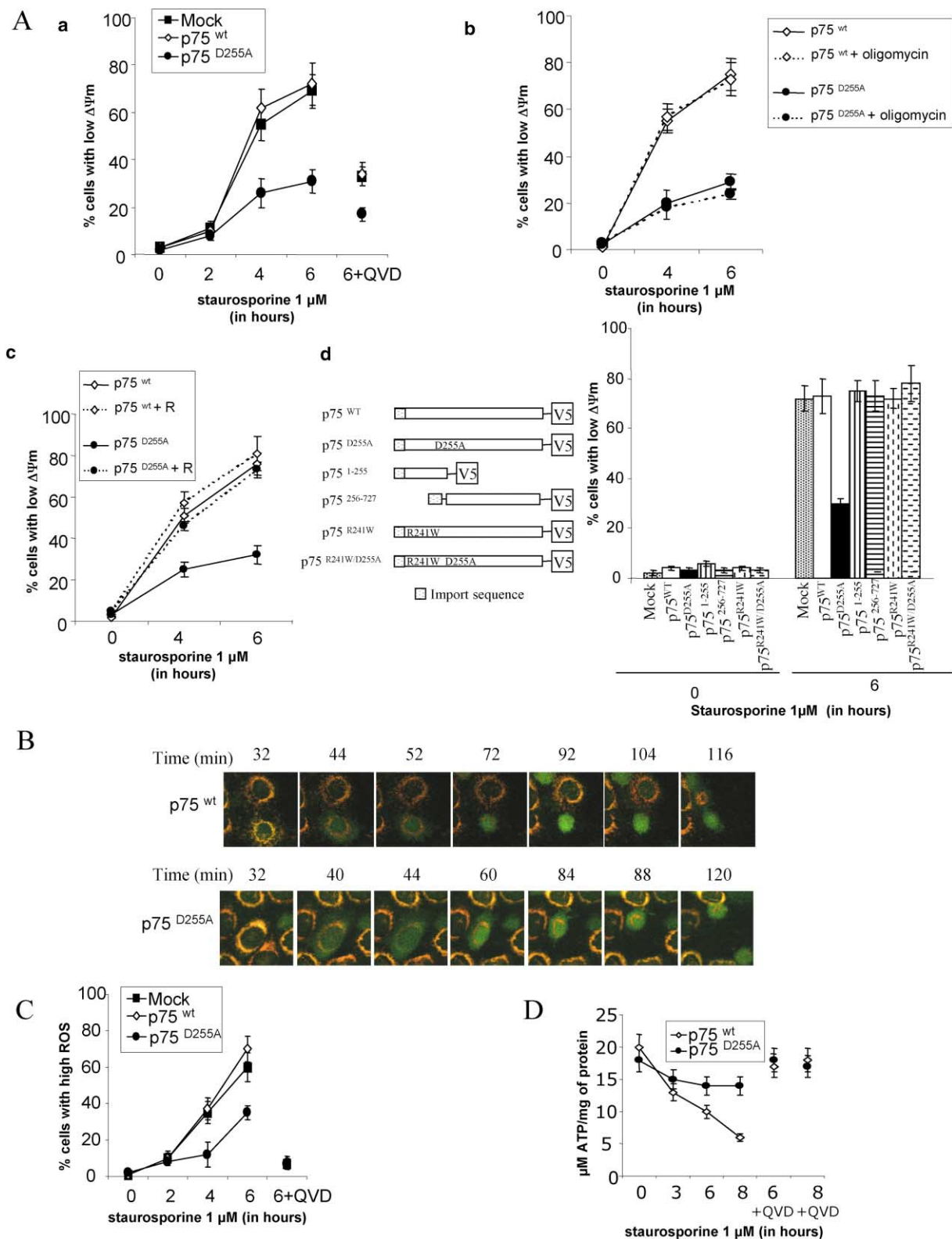


Figure 4. Expression of p75^{D255A} Preserves Mitochondrial Functions during Apoptosis

(A) (Aa) Effect of p75 cleavage on $\Delta\Psi_m$. HeLa cells were treated as indicated (Q-VD-OPH 20 μ M) and then incubated in the presence of TMRE for 30 min at 37°C, and $\Delta\Psi_m$ was measured by flow cytometry. (Ab) Lack of effect of oligomycin (10 μ g/ml) on sustained $\Delta\Psi_m$. (Ac) Effect of rotenone (2 μ M) on sustained $\Delta\Psi_m$. (Ad) HeLa cells were stably transduced with wt, D²⁵⁵A, fragments corresponding to the N- and C-terminal caspase cleavage products, and/or inactive (R²⁴¹W) forms of p75. Cells expressing each form were treated with staurosporine (6 hr) to induce apoptosis, and $\Delta\Psi_m$ was measured. (B) $\Delta\Psi_m$ (red) and cytochrome c-GFP (green) during Actinomycin D-induced apoptosis in HeLa cells

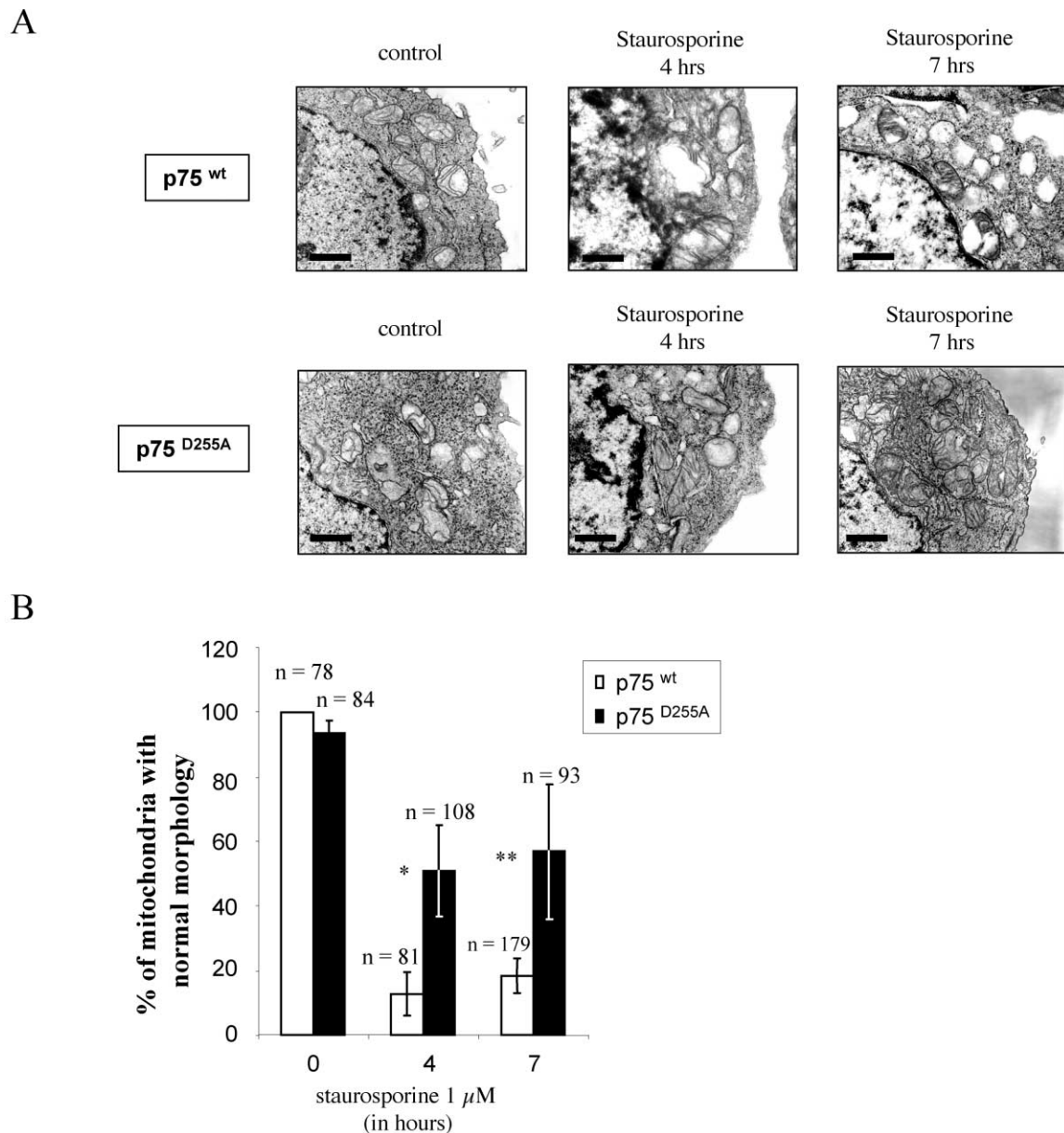


Figure 5. Expression of p75^{D255A} Delays Loss of Mitochondrial Integrity Occurring during Apoptosis

(A) HeLa cells were treated with 1 μM staurosporine for the indicated times, and ultrastructural morphology was visualized by electron microscopy. Typical morphologies are shown for each condition. Scale bar, 1 μm.

(B) Quantification of mitochondria with normal morphology. The number of mitochondria present in untreated p75^{wt}-expressing cells was set as 100%. n represents the number of mitochondria counted for each condition (10 cells were examined for each control condition; 12 cells for p75^{wt}, 4 hr; 14 cells for p75^{D255A}, 4 hr; 20 cells for p75^{wt}, 7 hr; and 13 cells for p75^{D255A}, 7 hr), *p = 0.000002, **p = 0.00008.

and Supplemental Movie S2) (while it is suggested at the population level, Figure 6B) is intriguing but may reflect the scoring—in Figure 6C, the time indicated is that of first appearance of Annexin V binding and does not reflect intensity of staining. Alternatively, this difference could be explained by the fact that Annexin V is

constitutively present in the media during the time-lapse experiments. As it has been shown that PS externalization is antagonized by the ATP-dependent phospholipid translocase in the plasma membrane (Hamon et al., 2000), the constitutive presence of Annexin V in the culture might interfere with this relocation of PS bound

expressing p75^{wt} or p75^{D255A} (see Supplemental Movie S1). (C) Effect of p75 cleavage on ROS production. Cells were treated as in (Aa), and ROS production was assessed using 2-HE by flow cytometry analysis. (D) Effect of p75 cleavage on ATP levels. Cells were treated as indicated and harvested, and total cellular ATP content was measured. Values in (A), (C), and (D) represent averages of three independent experiments.

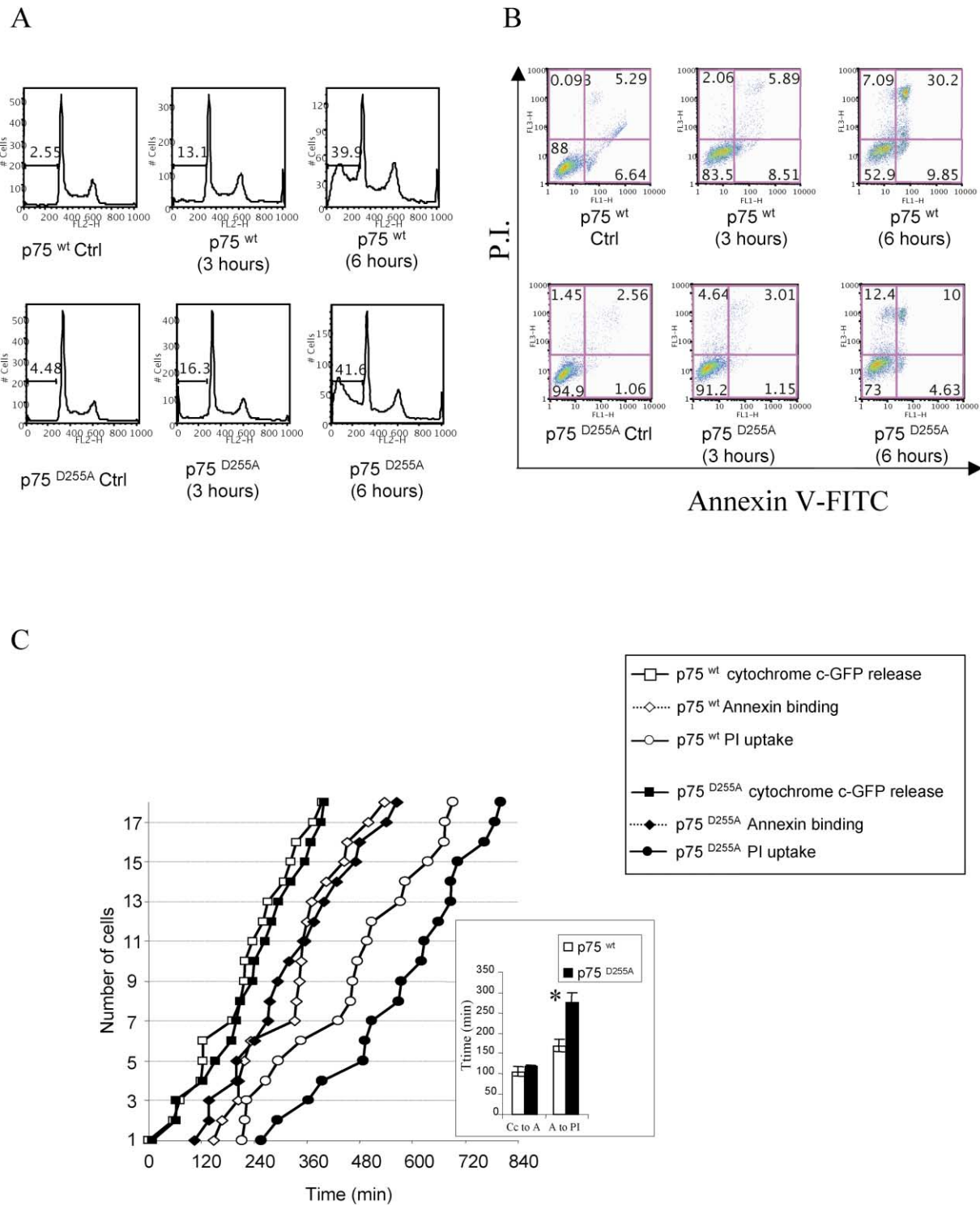


Figure 6. Expression of p75^{D255A} Influences Plasma Membrane Events during Apoptosis

HeLa cells were treated with 1 μ M staurosporine as indicated, then, for each sample, sub-G1 (A) and Annexin V-PI uptake (B) were assessed in parallel by flow cytometry analysis. (C) Cytochrome c release and plasma membrane events during apoptosis. Cells were treated as in (A) and examined by time-lapse confocal microscopy. The time of each event, cytochrome c release, PS externalization, and plasma membrane permeabilization (PI uptake) is indicated. The inset shows the average intervals \pm SEM (in min) between cytochrome c release and the externalization of PS (noted as Cc to A) and between PS externalization and PI uptake (A to PI). p75^{wt} (n = 46 cells) and p75^{D255A} (n = 36 cells), *p = 0.0004.

to Annexin V. In addition, one recent study showed that PS externalization during Fas-induced apoptosis is delayed by Bcl-2, which also sustained ATP levels in the cells (Uthaisang et al., 2003).

Our results show that, following cytochrome c release, activated caspases gain access to the intermembrane space where they cleave p75, resulting in disruption of complex I activity, loss of $\Delta\Psi_m$, production of ROS, and disruption of the mitochondrial structure. As a consequence, ATP levels drop, and plasma membrane integrity is lost. Therefore, p75 is a major target of executioner caspases contributing to the death of the cell.

Discussion

During apoptosis, mitochondria can play important roles both upstream and downstream of caspases. While the role of mitochondria in caspase activation is fairly well characterized, the effects of these proteases on mitochondria and how this influences the process of cell death have been far less clear. In our studies, we have identified one key caspase substrate accessible to the mitochondrial intermembrane space, the 75 kDa subunit (NDUFS1/NUAM) of complex I, and examined the effects of its cleavage by caspases during apoptosis. Our results show that the cleavage of p75 by caspases is responsible for disruption of electron transport (Figures 3D and 3E) and $\Delta\Psi_m$ (Figure 4A), leading to production of ROS (Figure 4C), loss of ATP production (Figure 4D), and mitochondrial damage (Figure 5).

Not surprisingly, preserving mitochondrial functions and integrity does not appear to affect DNA fragmentation during apoptosis, which is mediated by caspase cleavage of ICAD to release the active nuclease CAD (Enari et al., 1998). In contrast, the effect of p75 cleavage on plasma membrane events is intriguing. Cells expressing the noncleavable p75^{D255A} showed delays in persistent PS externalization and loss of plasma membrane integrity (Figure 6, Supplemental Figures S7 and S4C, and Supplemental Movie S2). One possibility is that the production of ROS upon p75 cleavage contributes to these plasma membrane events. Indeed, lipid peroxidation during apoptosis has been implicated in PS externalization (Arroyo et al., 2002; Matsura et al., 2002). Alternatively (or in addition), the effects may be a consequence of the maintenance of ATP in the dying cells. The lipid transporter that moves PS from the outer to the inner leaflet of the plasma membrane is ATP dependent (Gleiss et al., 2002), and it may be that its activity serves to counteract (at least partially) the scrambling of PS following caspase activation. Sustaining ATP levels would also support a variety of ion pumps in the plasma membrane, contributing to the maintenance of membrane integrity.

In our studies, the expression of noncleavable p75^{D255A} produced only partial protection of $\Delta\Psi_m$ in intact cells undergoing apoptosis (Figure 4A). Mitochondrial morphology was also only partially (albeit highly significantly) protected (Figure 5B). There are several likely reasons for this. First, the process of apoptosis in intact cells is stochastic, with different cells entering apoptosis at different times, making analysis of kinetics difficult (for this reason, we selected cells for ultrastructural as-

essment that showed similar chromatin condensation; Supplemental Figure S6). Caspase-independent effects can also contribute to the differences between the numerous processes occurring during apoptosis as opposed to the broken cell model (Goldstein et al., 2000). In addition, caspase-induced loss of complex II activity (Ricci et al., 2003a) should further contribute, since this complex is responsible for some of the electrons that enter the respiratory chain and is necessary for continued function of the citric acid cycle. Finally, while expression of p75^{D255A} is restricted to mitochondria in the stable lines, we do not know how many of the complexes in these cells incorporate the mutant versus the endogenous wt protein. Mitochondria with higher levels of the native complex may represent those that show disruption during apoptosis in the p75^{D255A}-expressing cells (approximately 50% of mitochondria per cell were observed to be disrupted in our ultrastructural analysis of these cells, consistent with this idea). At present, we do not know which of these explanations is most likely.

It might be argued that if a cell dies as a consequence of engaging the mitochondrial pathway, the precise features of the death controlled by caspase cleavage of a specific substrate are not of particular physiological significance. Indeed, inhibition of caspase activation fails to sustain clonogenicity of cells induced to undergo apoptosis (Amarante-Mendes et al., 1998), and cells can undergo a caspase-independent death (Jaattela and Tschopp, 2003). In cases such as the inhibition of oncogenic transformation, the kinetics and precise features of death may be irrelevant, provided that the cell does indeed die. However, the kinetics of cell death may be crucial in other cases. In development, a delay in death of a population of cells can lead to defects in morphogenic events, and this effect might explain the partial penetrance of the APAF-1 phenotype in cranio-facial development (Cecconi et al., 1998). Similarly, the rate of death and clearance of cells infected with viruses or other intracellular parasites might be expected to have important ramifications for the spread of the infection. In this regard, it is not unreasonable to speculate that the rapid loss of ATP production and generation of ROS caused by caspase cleavage of p75 contributes to the inhibition of parasite replication and perhaps parasite destruction in the dying cell. Rapid clearance upon PS externalization would further restrict the spread of the parasite. Therefore, caspase targeting of p75 to destroy complex I function is likely to be of importance in such settings.

The p75 subunit of complex I is highly conserved, and all animals examined maintain the caspase cleavage site we have identified in this molecule. However, this cannot be taken as proof (or even a suggestion) that this site is maintained by selection pressure for caspase cleavage of this protein during apoptosis, since the site is also present in *Arabidopsis* and in some bacteria (but absent in many others) that, as far as we know, do not employ caspases for cell death. Further, mitochondrial outer membrane permeabilization, which we have observed is necessary for this effect, does not appear to occur during apoptosis in *Drosophila* cells, and, indeed, $\Delta\Psi_m$ is sustained in these cells until very late in the apoptotic process (Zimmermann et al., 2002). The permeabilization of the mitochondrial membrane, followed

by caspase-mediated disruption of electron transport cleavage of p75, may therefore be restricted to a subset of animals, including the vertebrates.

Experimental Procedures

Proteins and Reagents

Carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) (10 μ M), cytochrome c 100 μ M, all the substrates and inhibitors of the electron transport chain (see below), and all the reagents were from Sigma (St. Louis, MO) unless stated otherwise. Tetramethylrhodamine ethyl ester (TMRE) was from Molecular Probes. Recombinant Bcl-xL- Δ C and caspase-3 were generated in bacteria as previously described (Bossy-Wetzel and Green, 1999). Bcl-xL- Δ C was used at 20 μ g/ml. Caspase-free 125 I-Bid was generated as described (von Ahsen et al., 2000). Antibodies to the following proteins were used: PARP (clone 7D3-6, BD Biosciences), V5 (Invitrogen), p39 (clone 20C11), p30 (clone 3F9), p20 (clone 20E9), p17 (clone 21C11), p15 (17G3) (all complex I subunit antibodies were from Molecular Probes), actin (clone C4, ICN Biomedicals), cytochrome c (clone 7H18.2C12; Pharmingen), caspase-3 (sc-7148, Santa Cruz), and hsp60 (clone LK-1, Stressgen). Rabbit antisera against the 75 kDa bovine complex I subunits were from Dr. John E. Walker.

Diagonal Gels

Mitochondria were isolated from mouse liver (see below), and, in order to increase the gel resolution, soluble or membrane-associated proteins were further separated using Triton X-114 (Bordier, 1981) or sodium carbonate (Fujiki et al., 1982), respectively.

Isolation of Soluble Mitochondrial Proteins

1 mg of mouse liver mitochondria was resuspended in 200 μ l of 10 mM Tris, 150 mM NaCl, 1% Triton X-114 and incubated 10 min at 0°C. The sample was loaded on the top of a sucrose fraction (300 μ l of 6% sucrose in 10 mM Tris, 150 mM NaCl, and 0.06% Triton X-114) and incubated for 3 min at 30°C, then centrifuged at 300 \times g at RT in a swinging bucket. The upper aqueous phase containing the soluble mitochondrial proteins was used.

Isolation of Membrane-Associated Proteins

Mitochondria were suspended at 1 mg/ml in 100 mM sodium carbonate (pH 11.5) and incubated 30 min at 0°C. The suspensions were centrifuged at 50,000 rpm for 1 hr at 4°C. The pellet corresponding to the membrane-associated proteins was dissolved in 2% SDS. In both cases, 400 μ g of proteins was resolved by SDS-PAGE 12% (first dimension). After migration, the lane containing the protein was excised and soaked in 40% EtOH and 10% Acetic acid for 10 min, in EtOH 30% for 10 min, and then in ultrapure water for 2 \times 10 min. The lane was then air dried (15–30 min) and soaked in buffer A (50 mM Tris-HCl, 150 mM NaCl, DTT 10 mM) with or without 50 μ g of active recombinant caspase-3 and incubated overnight at 37°C. The lane was washed in water to remove the excess protease and then incubated in loading buffer (50 mM Tris [pH 6.8], 2% SDS, 0.1% bromophenol blue, 10% glycerol, 2.5% β -mercaptoethanol) and incubated for 10 min at 95°C. After cooling, the lane was then loaded on a second acrylamide gel (12%) and resolved by SDS-PAGE. After migration, the gel was stained using GelCode Blue Stain Reagent (Pierce). Cleaved proteins, which are located under the diagonal, were excised from the gels and identified by MALDI-TOF (by the Molecular Analysis Facility of the Burnham Institute, La Jolla, CA.).

Cell Culture and Induction of Apoptosis

HeLa and H1299 cells were cultured in DMEM (GIBCO BRL) supplemented with 2 mM glutamine, 200 μ g/ml penicillin, 100 μ g/ml streptomycin sulfate, and 10% FBS. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂ in the presence of 1 μ g/ml of G418 for stably transfected cell lines. For passage, cells were incubated in 0.25% trypsin (GIBCO BRL), washed, and subcultured in growth media. To induce death, cells were preincubated or not for 15 min with 20 μ M Q-VD-OPH (ICN) and treated with staurosporine or actinomycin D (as indicated).

Cytochrome c-GFP Release Assay

Cells expressing cytochrome c-GFP were treated as indicated and then permeabilized with digitonin as described in the additional

experimental procedures (see Supplemental Data). The loss of GFP fluorescence corresponding to the cytochrome c released was assessed by flow cytometry analysis in FL-1 (Goldstein et al., 2000; Waterhouse et al., 2001). When cytochrome c-GFP and $\Delta\Psi$ m were recorded at the same time (Supplemental Figure S5A), cells were first incubated in the presence of 50 nM TMRE (30 min at 37°C), then permeabilized, washed, and analyzed by flow cytometry in the presence of cytochrome c (100 μ M). During permeabilization and washes, cells were maintained in the presence of TMRE (50 nM).

Oxygen Electrode Measurement

Two independent Clark oxygen electrodes (Instech Laboratories, Plymouth Meeting, PA) with two independent thermostatted chambers were used to analyze two samples in parallel. HeLa cells stably expressing the wt or the D²⁵⁵A form of p75 were incubated or not with 1 μ M staurosporine for 6 hr as indicated. Cells were then digitonin permeabilized (see Supplemental Data) and washed with respiration buffer (RB = 250 mM sucrose, 2 mM EDTA, 30 mM KH₂PO₄, 5 mM MgCl₂, 50 mM Tris [pH 7.4]) In Figures 3Dc and 3Dd, RB contained 2 mM ADP. The permeabilized cells were injected into the chambers containing 600 μ l of air-saturated RB prewarmed at 37°C. To rule out an effect of dilution of cytochrome c, all measurements of apoptotic cells were performed in the presence of 100 μ M cytochrome c. Substrates and inhibitors were added in the following order and final concentration: 5 mM malate, 5 mM glutamate, 2 μ M rotenone, 5 mM succinate, 1 μ M antimycin A. Oxygen concentration was calibrated with air-saturated buffer, assuming 390 ng atoms of oxygen/ml of buffer (Schulze-Osthoff et al., 1992). Rates of oxygen consumption are expressed as ng atoms of oxygen/min/mg of proteins.

Measurement of $\Delta\Psi$ m, ROS, and Apoptosis

Cells were treated as indicated, harvested, and washed in PBS. For assessment of Annexin V binding, cells were resuspended in 200 μ l of Annexin buffer (Hepes 10 mM, NaCl 150 mM, KCl 5 mM, MgCl₂ 1 mM, CaCl₂ 1.8 mM) containing 1/200 Annexin V-FITC (Calbiochem). Except for confocal studies (see below), $\Delta\Psi$ m was measured using TMRE (50 nM) in mitochondrial isolation buffer (MIB: 220 mM mannitol; 68 mM sucrose, 10 mM Hepes-KOH [pH 7.4], 70 mM KCl, 1 mM EGTA, 1 mM PMSF, and 2 μ M aprotinin; when permeabilized cells were examined, 100 μ M cytochrome c was added). ROS were measured using 2 μ M of dihydroethidium (2-HE) in MIB buffer. For sub-G1 analysis, after treatment, cells were washed and resuspended in 100 μ l of PBS, and the cells were fixed by addition of 900 μ l of EtOH 70% in PBS and incubated overnight at 4°C. Cells were washed in PBS and resuspended in 200 μ l of PBS plus 200 μ l of DNA-extracting solution (Na₂HPO₄ 0.2M + Citric Acid 0.1 M [pH 7.8]), then incubated for 10 min at 37°C. After centrifugation, cells were resuspended in PBS containing 100 μ g/ml of RNase A and 40 μ g/ml of propidium iodide. In all cases, cells were incubated for 30 min at 37°C in the dark. Analysis was made by flow cytometry. TMRE and 2-HE was measured in FL-2, Annexin V-FITC in FL-1, and propidium iodide (PI, 0.5 μ g/ml added at the last minute to the sample) in FL-3 or in FL-2 for sub-G1.

Additional Experimental Procedures

Methods for cloning, mutation, and retroviral transduction, siRNA, in vitro transcription and translation, in vitro cleavage by caspase-3, Western blotting, isolation of mitochondria, mitoplast isolation, coimmunoprecipitations, immunostaining, cell permeabilization, ATP measurements, confocal and electron microscopy, and statistics are provided in Supplemental Data.

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