The role of ARK in stress-induced apoptosis in *Drosophila* cells

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The molecular mechanisms of apoptosis are highly conserved throughout evolution. The homologs of genes essential for apoptosis in *Caenorhabditis elegans* and *Drosophila melanogaster* have been shown to be important for apoptosis in mammalian systems. Although a homologue for CED-4/apoptotic protease-activating factor (Apaf)-1 has been described in *Drosophila*, its exact function and the role of the mitochondrial pathway in its activation remain unclear. Here, we used the technique of RNA interference to dissect apoptotic signaling pathways in *Drosophila* cells. Inhibition of the *Drosophila* CED-4/Apaf-1–related killer (ARK) homologue resulted in pronounced inhibition of stress-induced apoptosis, whereas loss of ARK did not protect the cells from Reaper- or Grim-induced cell death. Reduction of DIAP1 induced rapid apoptosis in these cells, whereas the inhibition of DIAP2 expression did not but resulted in increased sensitivity to stress-induced apoptosis; apoptosis in both cases was prevented by inhibition of ARK expression. Cells in which cytochrome c expression was decreased underwent apoptosis induced by stress stimuli, Reaper or Grim. These results demonstrate the central role of ARK in stress-induced apoptosis, which appears to act independently of cytochrome c. Apoptosis induced by Reaper or Grim can proceed via a distinct pathway, independent of ARK.

**Introduction**

Apoptosis or programmed cell death is an essential physiological process required for normal development and maintenance of tissue homeostasis in both invertebrates and vertebrates (Jacobson et al., 1997; Raff, 1998; Vaux and Korsmeyer, 1999). However, apoptosis is also involved in a wide range of pathological conditions, including neurodegenerative, cardiovascular, and immunological diseases. The molecular mechanisms of apoptosis are highly conserved throughout evolution. In the nematode *Caenorhabditis elegans*, three of the essential components of the apoptotic pathway are *ced-3*, *ced-4*, and *ced-9* (Yuan and Horvitz, 1992; Miura et al., 1993; Hengartner and Horvitz, 1994). Homologs of these genes have been found in various species. The key mediators in this cell-killing pathway are a family of aspartate-specific cysteine proteases, termed caspases, that includes several mammalian proteases and CED-3 of *C. elegans* (Miura et al., 1993). Long prodomain caspases (initiator caspases) function as signal integrators for apoptotic or proinflammatory signals and contain sequence motifs that promote their interaction with activator molecules (caspase recruitment domain or death effector domain [Hofmann et al., 1997; Ashkenazi and Dixit, 1998]). The apoptotic initiator caspases generally act upstream of the small prodomain-containing executioner caspases, which orchestrate the death of the cell (Nicholson and Thornberry, 1997; Ashkenazi and Dixit, 1998). The activation of caspases is tightly controlled by both positive and negative regulators. Members of the inhibitor of apoptosis protein (IAP)* family can inhibit caspases and apoptosis in a variety of insect and vertebrate systems (Hawkins et al., 1998; Deveraux and Reed, 1999). Caspase activation is stimulated by a protein represented by *C. elegans* CED-4, *Drosophila melanogaster* apoptosis protease-activating factor (Apaf)-1–related killer (ARK; also known as Dark, Hac-1 or Dapaf-1), and mammalian APAF-1. There may be significant differences between the mechanisms for initiation of caspase activation in vertebrates and invertebrates. In *C. elegans*, CED-4 is activated and converted to an

*Abbreviations used in this paper: Apaf, apoptotic protease-activating factor; ARK, Apaf-1–related killer; dsRNA, double-stranded RNA; GFP, green fluorescent protein; Hid, head involution defective; IAP, inhibitor of apoptosis protein; PI, propidium iodide; RNAi, RNA interference; SL2, Schneider cell line 2; TMRE, tetra-methyl-rhodamine-ethyl ester; zVAD-fmk, benzoxycarbonyl-Val-Ala-Asp-fluoromethylketone.*
Figure 1.  **Stress or macromolecular synthesis inhibitor induce apoptosis in Drosophila cells.** SL2 cells were treated with UV (90 mJ/cm²), cycloheximide (CHX, 10 μM), or actinomycin D (ActD, 1 μM) in the presence or absence of zVAD-fmk. (A) Exposure of phosphatidylserine on the cell surface of Drosophila cells indicated by binding of annexin V–FITC after 6 h of treatment. (B) FACS® analysis of DNA fragmentation indicated by subdiploid DNA-containing nuclei after 8 h of treatment. Data are representative for three independent experiments. (C) Caspase activation indicated by hydrolysis of the fluorogenic substrate DEVD-AFC after 8 h of treatment. Error bars indicate SD. (D) Representative images showing Δψm, phosphatidylserine exposure, and plasma membrane integrity in SL2 cells after treatment with UV. Cells are stained with the potentiometric mitochondrial dye TMRE (red), annexin V–FITC (green), and propidium iodide (magenta). Time in hours. Bar, 10 μm.
“active” form when the association between CED-4 and the antiapoptotic Bcl-2 homologue CED-9 is disrupted by EGL-1, a BH3-containing protein (Conradt and Horvitz, 1998). However, in mammals Apaf-1 requires cytochrome c released from mitochondria (Zou et al., 1997), which binds to the WD repeats at the COOH terminus of Apaf-1. Through the formation of an “apoptosome” (consisting of cytochrome c, Apaf-1, and pro-caspase-9) caspase-9 becomes activated, which subsequently activates the effector caspase-3 (Li et al., 1997). Because Apaf-1 requires cytochrome c as a cofactor for caspase activation in vitro, it has been demonstrated that the release of cytochrome c from mitochondria into the cytosol is a critical regulatory step, which is controlled by anti- and proapoptotic members of the Bcl-2 family (Kluck et al., 1997; Li et al., 1997; Yang et al., 1997; Green and Reed, 1998).

Apoptosis in Drosophila involves three death-inducing genes, head involution defective (hid), reaper, and grim (Abrams, 1999), which are required for essentially all normally occurring cell deaths in Drosophila embryogenesis (White et al., 1994; Grether et al., 1995; Chen et al., 1996). Reaper, Hid, and Grim are thought to induce apoptosis by binding to and inactivating the IAPs (for review see Miller, 1999). Each of these proteins induces apoptosis in mammalian cells (Claveria et al., 1998; McCarthy and Dixit, 1998; Haining et al., 1999), but sequence homologs have not been identified in other organisms.

Apoptosis induced by DNA damage in vivo in C. elegans and Drosophila appears to require transcriptional up-regulation of EGL-1 (Gartner et al., 2000) and Reaper (Nordstrom et al., 1996), respectively. In contrast, the demonstration that macromolecular synthesis inhibitors induce apoptosis in mammalian cells (Martin et al., 1990) enforced the idea that the components of the apoptotic program preexist in cells, awaiting a trigger. Therefore, we have explored apoptosis induced by stress or macromolecular synthesis inhibitors in Drosophila cells and examined the different roles of ARK, cytochrome c, and IAPs in the process.

Results

Characterization of apoptosis in Drosophila cells

We have examined apoptosis in Drosophila Schneider cell line 2 (SL2) induced by stress or macromolecular synthesis inhibitors. An early event in mammalian apoptosis is the exposure of phosphatidylserine on the cell surface, which can be analyzed by binding of annexin V–FITC and FACS®. Like mammalian cells, Drosophila Schneider cells expose phosphatidylserine after treatment with UV, cycloheximide, or actinomycin D (Fig. 1 A). The phosphatidylserine externalization is caspase dependent, since it can be prevented by benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk). Drosophila cells also show DNA fragmentation (quantified by determination of subdiploid nuclei [Fig. 1 B]), and caspase activation (measured by cleavage of the fluorescent substrate DEVD-AFC [Fig. 1 C]) after treatment with UV or macromolecular synthesis inhibitors. Both can be inhibited by treatment with zVAD-fmk. The topoisomerase II inhibitor etoposide also induced apoptosis but was much less efficient than these agents (unpublished data). Models of downstream apoptotic events in vertebrates begin with the release of cytochrome c and resultant caspase activation. This is followed by phosphatidylserine externalization and loss of plasma membrane integrity. We used time-lapse confocal microscopy to determine the order of events during apoptosis in Drosophila cells. Single cell analysis of Drosophila SL2 cells showed that the exposure of phosphatidylserine on the cell surface, as indicated by the binding of green annexin V–FITC, occurred before the loss of mitochondrial potential (ΔΨm) as detected by loss of staining by the red dye tetra-methyl-iodoacetamide-ethyl ester (TMRE). Subsequently, the mitochondria lost their membrane potential and the cells lost their plasma membrane integrity as detected by uptake of propidium iodide (PI) (Fig. 1 D). All cells observed to undergo apoptosis by this assay showed this order of events. In a separate experiment (unpublished data), cells were assessed by FACS® for staining with TMRE versus annexin V–FITC after treated with UV. The percent of annexin V®/H11006® and caspase activity or may represent a slower ARK-independent pathway leading to caspase activation and death.

Silencing of ARK inhibits stress-induced apoptosis in Drosophila cells

To determine whether decreased levels of ARK gene expression would affect apoptosis in Drosophila SL2 cells, we treated cells with double-stranded RNA (dsRNA) synthesized from the ARK cDNA (see Materials and methods). ARK mRNA levels decreased significantly 1 d after treatment with dsRNA, but the maximum decrease was found 3 d after treatment as determined by real time PCR with ARK-specific primers (Fig. 2 A). Since an ARK antibody was not available, we transfected Schneider cells with myc-tagged ARK under the control of a metallothioneine promoter and found profound suppression of ARK protein expression after subsequent treatment with ARK dsRNA (Fig. 2 B). Treatment of ARK-deficient SL2 cells with UV light resulted in complete suppression of caspase activation over a time course of 12 h (Fig. 2 C) compared with control cells. These cells were also resistant to varying doses of UV light (Fig. 2 D).

To further extend our observations, we used other apoptosis-inducing stimuli for treatment of the ARK-deficient cells. These cells were not only protected against UV-induced apoptosis but also against apoptosis induced by cycloheximide and actinomycin D as assessed by morphological features (Fig. 3 A), DNA fragmentation (Fig. 3 B), and caspase activation (Fig. 3 C). However, although the cells were resistant over this period of time some caspase-dependent cell death was seen after 24 h. As shown in Fig. 3 E, pretreatment with ARK dsRNA afforded some but not complete protection 24 h after UV treatment, and this could be inhibited by addition of the caspase inhibitor zVAD-fmk. At this time, a small amount of caspase activity was detected in the ARK dsRNA plus UV-treated cells (unpublished data). It is possible that this late caspase-dependent death is a consequence of low residual levels of ARK activity or may represent a slower ARK-independent pathway leading to caspase activation and death.
Down-regulation of DIAP1 expression leads to caspase activation and cell death, whereas down-regulation of DIAP2 expression sensitizes Drosophila cells to apoptosis

DIAP1 and DIAP2 belong to the family of IAPs, the only known cellular caspase inhibitors (Deveraux and Reed, 1999). The Drosophila genome encodes four IAPs, including DIAP1, DIAP2, Deterin, and dBruce. Overexpression of DIAP1 or DIAP2 in the eye suppresses normally occurring cell death or death due to overexpression of Reaper or Hid (Hay et al., 1995), and elimination of DIAP1 function results in global early embryonic cell death (Wang et al., 1999). Treatment of SL2 cells with DIAP1 dsRNA resulted in decreased DIAP1 mRNA expression after 1 d (Fig. 4 A). These cells showed caspase activation (Fig. 4 B) and died spontaneously (Fig. 4 C). Pretreatment with ARK dsRNA prevented caspase activation (Fig. 4 B) and apoptosis (Fig. 4 C) induced by the DIAP1 dsRNA.

We also examined the effect of inhibition of DIAP2 expression on apoptosis. After treatment with DIAP2 dsRNA, DIAP2 expression decreased over a period of 2 d as determined by real time PCR (Fig. 4 A). These cells showed an enhanced susceptibility to apoptosis induced by UV, cycloheximide, or actinomycin D, which could be prevented by cotreatment of these cells with ARK dsRNA (Fig. 4 D). Therefore, apoptosis that is normally inhibited by DIAP1 or DIAP2 is dependent on the function of ARK in these cells.

Drosophila cell mitochondria do not release cytochrome c or lose their membrane potential (ΔΨm) during stress-induced apoptosis

Previous reports show that in contrast to the mammalian model of apoptosis cytochrome c is not released during Reaper- or Grim-induced apoptosis in Drosophila cells but instead cofractionates with mitochondria (Varkey et al., 1999). Therefore, we investigated the role of cytochrome c in stress-induced apoptosis. SL2 cells were treated with UV, cycloheximide, or actinomycin D for 8 h and assayed for cytochrome c in the mitochondrial and cytosolic fractions. These cells showed no difference in cytochrome c distribution. There was no evidence for the transit of cytochrome c to the cytosol as a correlate of apoptosis (Fig. 5 A). We also stained the cells with the mitochondrial dye TMRE, which indicates mitochondrial integrity and membrane potential, and noted that some of the cells showed annexin V binding along with TMRE staining, indicating that although apoptotic these cells still maintain mitochondrial membrane potential (Figs. 5 B, and 1 D). It has been shown recently that loss of ΔΨm occurs shortly after...
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mitochondrial cytochrome c release in mammalian cells (Waterhouse et al., 2001), whereas binding of annexin V is a downstream event, which requires caspase activation (Goldstein et al., 2000). These observations suggest that the release of cytochrome c is not an early event in *Drosophila* apoptosis and loss of mitochondrial integrity occurs only after caspase activation and phosphatidylserine exposure.

**Down-regulation of cytochrome c does not protect cells from stress-induced apoptosis**

Therefore, we sought to determine if cytochrome c is a part of apoptotic signaling pathways in *Drosophila*. Two cytochrome c genes, DC3 and DC4, have been described in *Drosophila melanogaster* (Limbach and Wu, 1985), but studies at the level of protein (Inoue et al., 1986) and at the level of RNA (Limbach and Wu, 1985) suggest that DC4 is either the predominant or only form of actively expressed product. After treatment with cytochrome c (DC4), dsRNA, a >200-fold down-regulation of cytochrome c protein expression occurred on the third day (Fig. 6 A) as detected by immunoblot with an anti–human cytochrome c that cross reacts with *Drosophila* DC4 (Varkey et al., 1999). Since the region of DC4 we used for the generation of dsRNA is 71% identical to the same region in DC3, we suspected that the DC4 dsRNA would also inhibit DC3 expression, and therefore we examined levels of DC3 mRNA in the dsRNA-treated cells. Real time PCR of DC3 mRNA revealed almost complete down-regulation of DC3 mRNA levels after treatment with dsRNA (Fig. 6 B). Therefore, the treatment with the DC4 dsRNA inhibited expression of both forms of cytochrome c.

Although these cells showed pronounced down-regulation of cytochrome c protein expression, there was no change in their response to stress-induced apoptosis. The treated cells died after treatment with UV, cycloheximide, or actinomycin D comparably to control cells, showing the morphological features (Fig. 6 C), DNA fragmentation (Fig. 6 D), and caspase activation (Fig. 6 E) of an apoptotic cell. However, cells treated with both ARK and CYTC dsRNA were protected from stress-induced apoptosis (unpublished data). Thus, lack of cytochrome c does not affect apoptosis in general. These results strongly suggest that cytochrome c is not important for apoptotic signaling in *Drosophila* cells.

**Neither silencing of ARK nor cytochrome c prevents Reaper- or Grim-induced cell death**

Previous studies on *Drosophila* Schneider cells have shown that expression of Reaper or Grim triggers apoptosis in cultured cells or in transgenic animals. They kill by inactivating the antiapoptotic IAP molecules, which directly bind and inhibit several *Drosophila* caspases. Since it has been claimed that Reaper induces apoptosis by release of cytochrome c (Evans et al., 1997) and that Reaper or Grim both are able to activate ARK (Rodriguez et al., 1999), we tested these hypotheses in our system. Transfection of Schneider cells with...
Reaper- or Grim-induced pronounced cell death after 24 h. However, neither pretreatment of the cells with ARK nor cytochrome c DC4 dsRNA could inhibit cell death induced by transient overexpression of Reaper (Fig. 7 A) or Grim (Fig. 7 B). In contrast, treatment of the cells with the broad spectrum caspase inhibitor zVAD-fmk prevented Reaper- or Grim-induced cell death (Fig. 7 A and B). These observations suggest that Reaper or Grim can induce caspase-dependent apoptosis independently of ARK or cytochrome c.

Debcl-induced cell death is neither dependent on caspases nor on ARK and cytochrome c in Drosophila cells

Drosophila possess a proapoptotic Bcl-2 family member, which is known as Drob-1 (Igaki et al., 2000), dBorg-1 (Brachmann et al., 2000), Debcl (Colussi et al., 2000), or dBok (Zhang et al., 2000). Debcl has been reported to induce apoptosis in Drosophila cells, which seems to be dependent on caspases and ARK (Colussi et al., 2000). In contrast to this, Drob-1–induced apoptosis has been described as caspase independent (Igaki et al., 2000). However, Debcl/Drob-1 seems to exert its function via the mitochondria as a localization to the mitochondrial membranes in Drosophila cells has been described (Igaki et al., 2000). Debcl/Drob-1–induced pronounced cell death in SL2 cells, which seemed to be caspase independent, since it could not be inhibited by zVAD-fmk (Fig. 8). In ARK dsRNA- or cytochrome c DC4 dsRNA-treated cells, transfection with Debcl/Drob-1–induced cell death, which argues that neither of them plays a requisite role in the signaling pathway of Debcl/Drob-1 leading to caspase-independent cell death.

Discussion

In this study, we have investigated apoptotic signaling pathways in Drosophila cells. Apoptosis in Drosophila is either a result of developmental activation of the cell death genes encoding Reaper, Hid, and Grim or it can be induced by the recently identified Apaf-1 homologue ARK. Although Reaper, Hid, and Grim exert their effect at least in part by inhibiting IAPs, which subsequently leads to activation of caspases, the role of ARK and the mechanism of its activation remains unclear. In this paper, we have shown that ARK is essential for stress-induced apoptosis in Drosophila cells. Down-regulation of ARK expression in Drosophila cells by dsRNA resulted in complete resistance to UV-, cycloheximide-, and actinomycin D–induced apoptosis (Igaki et al., 2000). However, Debcl/Drob-1 seems to exert its function via the mitochondria as a localization to the mitochondrial membranes in Drosophila cells has been described (Igaki et al., 2000). Debcl/Drob-1–induced pronounced cell death in SL2 cells, which seemed to be caspase independent, since it could not be inhibited by zVAD-fmk (Fig. 8). In ARK dsRNA- or cytochrome c DC4 dsRNA-treated cells, transfection with Debcl/Drob-1–induced cell death, which argues that neither of them plays a requisite role in the signaling pathway of Debcl/Drob-1 leading to caspase-independent cell death.
apoptosis. However, removal of ARK did not prevent Reaper- or Grim-induced apoptosis in Drosophila cells.

It has been shown previously that cytochrome c is not released during Reaper- or Grim-induced apoptosis in Drosophila cells (Varkey et al., 1999). To further investigate this issue in stress-induced apoptosis, we analyzed mitochondria and cytosol for cytochrome c content after treatment with UV, cycloheximide, or actinomycin D. Although these cells are apoptotic, cytochrome c remains in the mitochondrial fraction. Because cytochrome c in this fraction did not decrease and cytosolic cytochrome c did not appear in cells that were extensively undergoing apoptosis, we favor the interpretation that it is not released versus other possibilities (e.g., it may be released but degraded very rapidly).

In addition, apoptotic Drosophila cells expose phosphatidylserine on their cell surface, whereas their mitochondria nevertheless stain with TMRE, indicating membrane potential and mitochondrial integrity. With RNA interference (RNAi), we suppressed expression of both Drosophila cytochrome c proteins. These cells showed no resistance to apoptosis induced by stress stimuli, Reaper or Grim. Furthermore, ectopic expression in Drosophila cells with the proapoptotic Bcl-2 homologue Debcl/Drob-1–induced caspase-independent cell death, which did not depend on ARK or cytochrome c.

There is accumulating evidence that in vertebrate models of apoptosis, mitochondria play an essential role (Green and Reed, 1998) by releasing apoptogenic factors, such as cytochrome c (Kluck et al., 1997; Yang et al., 1997), AIF (Lorenzo et al., 1999), Smac/Diablo (Du et al., 2000; Verhagen et al., 2000), EndoG (Li et al., 2001), or HtrA2/Omi (Suzuki et al., 2001; Hegde et al., 2002; Martins et al., 2002; Verhagen et al., 2002) from the intermembrane space into the cytoplasm. In the case of cytochrome c, caspase activation occurs through the formation of an “apoptosome,” which consist of Apaf-1, cytochrome c, and procaspase-9.
Like its counterpart in mammals, the Apaf-1 homologue ARK seems to be essential for stress-induced apoptosis in Drosophila cells, since its removal completely suppressed UV-, cycloheximide-, and actinomycin D–induced apoptosis. Like Apaf-1, Drosophila ARK has a nucleotide binding site in the NH₂-terminal CED-4 domain, and like Apaf-1 but distinct from CED-4 the COOH-terminal domain contains two series of WD repeats, which are responsible for cytochrome c binding by mammalian Apaf-1 (Li et al., 1997; Zou et al., 1997). This WD domain seems to act as a negative regulator of ARK function, since a COOH-terminal truncation of the WD repeat region showed enhanced cell-killing activity (Rodriguez et al., 1999), and therefore it is likely that an activator interacts with this domain during apoptosis in Drosophila. Although binding between ARK and cytochrome c has been shown in vitro (Kanuka et al., 1999; Rodriguez et al., 1999), cytochrome c is not released from Drosophila mitochondria during stress-induced apoptosis and may therefore not be available for such binding. Furthermore, cytochrome c failed to induce caspase activation in insect cell extracts (Varkey et al., 1999; unpublished data), although in one study addition of cytochrome c plus dATP induced a small amount of caspase activation in an embryo extract (Kanuka et al., 1999). However, in that study there was a 2.5-fold induction of caspase activity, which contrasts with the 50–100-fold induction or more seen in vertebrate cell extracts. In our studies, down-regulation of cytochrome c protein expression did not inhibit apoptosis. Therefore, our data suggest that cytochrome c, in contrast to the mammalian model, is not an apoptogenic factor in Drosophila.

If cytochrome c does not activate ARK, how does it become activated? One possibility is that the form of ARK in these cells is constitutively active, as suggested for an alternatively spliced form of ARK called dApaf-1S, which lacks the WD domain (Kanuka et al., 1999). However, as reported by others (Kanuka et al., 1999), we were unable to identify this alternatively spliced form of ARK in SL2 cells by PCR (unpublished data). Alternatively, there is a possibility that another mitochondrial activator is released from mitochondria during apoptosis, although our data suggest permeabilization of the mitochondrial outer membrane is not involved, since mitochondria maintain their membrane potential during the early caspase-dependent stages of apoptosis. In contrast, Debcl/Drob-1 appeared to induce caspase-independent apoptosis in Drosophila cells, which may indicate an effect on mitochondrial function. In mammalian and yeast cells, Bax and Bak can induce cell death via mitochondrial dysfunction (Zha et al., 1996; Jurgensmeier et al., 1997; Cheng et al., 2001). It has been reported that Reaper, Hid, and Grim are upstream of ARK and genetically interact with it, since an ARK mutant substantially suppressed the "rough eye" phenotype induced by expression of Reaper, Hid, and Grim (Kanuka et al., 1999; Rodriguez et al., 1999), although another study failed to find such an effect (Zhou et al., 1999). When we transiently overexpressed Reaper and Grim in our system, the cells died even in the absence of ARK. This cell death was probably dependent on caspase activation, since it was inhibited by zVAD-fmk. Since signaling pathways might be dependent on the cell type, there could be a difference between eye cells and Schneider cells. Alternatively, the ARK-independent effect may be due to overexpression of Reaper or Grim. However, we can conclude that in certain settings of apoptosis Reaper and Grim are able to induce caspase-dependent cell death independently of ARK. Reaper, Hid, and Grim are thought to induce apoptosis by binding to and inactivating IAPs. ARK also seems to affect apoptosis regulated by IAPs, since its down-regulation can overcome enhanced
susceptibility of *Drosophila* cells to stress-induced apoptosis caused by removal of DIAP2. IAPs are important regulators of caspase function, and it has been suggested that simple removal of IAPs will lead to cell death. Treatment of *Drosophila* cells with DIAP1 dsRNA decreased mRNA levels significantly after 1 d, which caused spontaneous death of the cells. Silencing of ARK prevented DIAP1-induced death of the cells. Treatment with DIAP2 dsRNA did not have an effect on the viability of the cells but increased their susceptibility to stress-induced apoptosis, which is also ARK dependent. These results suggest that caspase activation controlled by DIAP1 and DIAP2 is ARK dependent in *Drosophila*. Since inhibition of DIAP1 expression appears to spontaneously allow caspase activation to manifest, it is likely that some level of ARK function leading to DIAP1-inhibited caspase activation may be constitutive in these cells.

Apoptosis in mammalian cells can occur independently of transcriptional and translational control (Martin et al., 1990). The protein synthesis inhibitor cycloheximide or the RNA synthesis inhibitor actinomycin D induced apoptosis in *Drosophila* cells, which is caspase dependent since it could be completely blocked with zVAD-fmk. These results argue that in accordance with the mammalian model, insect cells also have no absolute requirement for new RNA or protein synthesis for apoptosis to occur. In cells which do not require new protein synthesis to undergo apoptosis, effector molecules must be present already within the cell and are either compartmentalized within the cell such that they can do no damage until specifically released to do so or they are regulated in other ways. Our studies indicate that although ARK is probably not activated via the release of cytochrome c from mitochondria, the activation of this protein is an important step in stress-induced apoptosis in *Drosophila* cells.

**Materials and methods**

**Cell culture and induction of apoptosis**

SL2 cells were propagated in Schneider's *Drosophila* media (GIBCO BRL) supplemented with 10% FCS, 50 U/ml penicillin, and 50 µg/ml streptomycin at 25°C. Cells treated with double-stranded cytochrome c RNA were cultured in medium also containing 50 µg/ml uridine and 110 µg/ml pyruvate (King and Attardi, 1989). Induction of apoptosis, SL2 cells were treated with actinomycin D (1 µM), cycloheximide (10 µM), or UVC ultraviolet C (45–180 mJ/cm²). For UV treatment, cells were washed in PBS and irradiated with UV light in PBS. The PBS was aspirated, and the medium was replaced. Caspase inhibition was achieved by including 100 µM zVAD-fmk (Enzyme Systems) in the medium where indicated.

**dsRNA synthesis**

Drosophila ARK, cytochrome c, DIAP1, and DIAP2 were amplified by PCR from a Schneider oligo-dT plasmid library (Berkeley *Drosophila* Genome Project). Each primer used in the PCR contained a T7 polymerase binding site (GAATTAATACGACTCACTATAGGGAGA) followed by sequences specific for ARK (sense primer, CGCCGACCCGGAAAAATGTATTTGATGATGGC and antisense primer, CGGAGGTCTAGCAGATATGCTTG), cytochrome c (sense primer, ATGGGCCGTTCTCGGTGTTGATGTTGGAAAGA and antisense primer, TTACCTTGTCGCCGACCTTCAGGTGAGG), DIAP1 (sense primer, ATGCGACCTGTGATGATTGCCTCTT and antisense primer, TCCGAGGATGGGCTAGGCGACATC), and DIAP2 (sense primer, ATGCGGACCTGTTGATGATTGCCTCTT and antisense primer, TCCGAGGATGGGCTAGGCGACATC). The PCR product was purified by ethanol precipitation and used as a template by using the MEGASCRIPT T7 transcription kit (Ambion) to produce dsRNA. The dsRNA samples were ethanol precipitated and resuspended in water. 5 µg of dsRNA were analyzed by 1% agarose gel electrophoresis to ensure that the majority of the dsRNA existed as a single band. The dsRNA was stored at −80°C.

**Conditions for RNAi in *Drosophila* cell culture**

RNAi methods were essentially as described (Clemens et al., 2000). In brief, SL2 cells were diluted to a final concentration of 10⁶ cells/ml in *Drosophila* Expression System serum-free medium (Invitrogen). dsRNA was added directly to the medium to a final concentration of 10 µg. This was followed immediately by vigorous agitation. The cells were incubated for 30 min at room temperature followed by addition of 2 ml Schneider's medium containing FCS. The cells were incubated for the indicated amount of time to allow for turnover of the promoter.

**Transfection of SL2 cells**

For cell death assays, 10⁶ cells were seeded into 60-mm dishes (Coming) in 2 ml of medium the day before transfection. Cells were transfected using Cellfectin reagent as per manufacturer's protocol (GIBCO BRL). For death assays, 10³ cells were cotransfected with 0.3 µg of pH-Gem or pH-Reaper (provided by H. Steller, Rockefeller University, New York, NY) and 0.5 µg of pE–green fluorescent protein (GFP) (provided by G. Jones, University of Kentucky, Lexington, KY). Where indicated, 100 µM zVAD-fmk was added to the cultures at the time of transfection. 24 h later, transfected cells were analyzed for the GFP reporter by FACSscan™. For experiments with copper-inducible pMT-Dark-I(−411)-myc (provided by J. Abrams, University of Texas Southwestern Medical Center, Dallas, TX) or pMT-debcl (provided by L. Dorsen, Institute of Medical and Veterinary Science, Adelaide, Australia), transfected cells were treated with 0.7 mM CuSO₄ 24 h after transfection to induce protein expression. At the indicated time, transfected cells were analyzed for the GFP reporter by FACSscan™. Cell survival was calculated as the percentage of GFP-positive cells in CuSO₄-treated cells relative to the percentage of GFP-positive cells in untreated cells.

**Reverse transcription real time PCR**

RNA was isolated from SL2 cells by Trizol (GIBCO BRL) extraction according to manufacturer’s instruction, and first strand cdNA was generated using 25 ng total RNA and Superscript™ reverse transcriptase (GIBCO BRL). Real time PCR™ was performed and AmpliTaQ Gold™ polymerase in a PE Biosystems 7300 thermocycler using SyBr Green detection protocol as outlined by the manufacturer. Sequence specific primers, each in a concentration of 50 nM, for ARK (sense primer, TTGGAAGAGGGGATACAAAGTG and antisense primer, TGGGAAATCGGCTGTGTATGAA), DIAP1 (sense primer, ATGCGACCTGTGATGATTGCCTCTT and antisense primer, TGCTGTGATGATTGCCTCTT), DIAP2 (sense primer, ATGCGACCTGTGATGATTGCCTCTT and antisense primer, CTGCTGTGATGATTGCCTCTT), DC3 (sense primer, TACCGCTTCCAAATGCGT and antisense primer, GCGCGACTTTGCA), and *Drosophila* actin primer (sense primer, GACCCCTGTACACCTTA and antisense primer, TCCGTAGTACGGGCACAC). All quantitative measurements were performed in triplicate and standardized relative to the internal control of actin mRNA for each sample.

**Western blotting**

Cells were dislodged by media and washed in PBS. The cells were incubated on ice for 20 min in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 100 µM PMSF, and complete protease inhibitor™ (Boehringer)). The homogenates were centrifuged at 14,000 g for 10 min, and the supernatants were stored at −80°C for Western blot. The protein content of the cell extracts was determined by the Bradford assay (Bio-Rad Laboratories). Protein from each sample was boiled for 5 min in Laemmli buffer and electrophoresed in individual lanes of 15% SDS–PAGE gels. The proteins were transferred to nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech) and Western blotted using anti–cytochrome c (clone 7H8.2C12; BD PharMingen), anti-actin (clone C4; ICN Biomedicals), or antitubulin (clone 4E1; Santa Cruz Biotechnology, Inc.) diluted 1:1,000. The immunoblotted proteins were incubated with HRP secondary antibodies (Amersham Pharmacia Biotech), and the signal was detected by ECL (Super Signal; Pierce Chemical Co.).

**Isolation of SL2 mitochondria**

Cells were harvested after the indicated treatment and time and washed in PBS twice, resuspended in extraction buffer (220 mM mannitol, 68 mM sodium succrose, 50 mM Pipes–KOH, pH 7.4, 80 mM KC1, 5 mM EGTA, 2 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol) including complete protease inhibitor™ (Boehringer) and lysed with 20 strokes of a Teflon homogenizer. Unbroken cells and nuclei were pelleted at 600 g for 5 min, and the supernatant was centrifuged for 10 min at 5,500 g to pellet the mitochondria. Mitochondrial pellet and supernatant were analyzed for cytochrome c content by SDS–PAGE and Western blotting using anti–cytochrome c (clone 7H8.2C12; BD PharMingen).
Apoptosis assays

Cells having undergone specific apoptotic events were detected by flow cytometry using a FACScan® (Becton Dickinson) with a 488-nm laser line and analyzed using Cell Quest software. Phosphatidylserine exposed on the outside of cells was determined by annexin V binding. In brief, cells were pelleted and resuspended in 100 μl of annexin V-FITC (Calbiochem) diluted 1:100 in annexin buffer (10 mM Hepes, 100 mM NaCl, 10 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂). Cells were incubated for 5 min, and 200 μl annexin buffer was added before FACScan® analysis. Annexin V-FITC fluorescence was detected in FL-1. Quantitation of subdiploid DNA containing nuclei was performed by detecting PI staining. In brief, cells were pelleted and fixed with ethanol at −20°C for 2 h. Cells were incubated for 30 min in PBS containing PI (50 μg/ml) and 1.5 μg/ml RNase Cocktail™ (Ambion). PI was detected in FL-3. Caspase activity was determined using the DEVD-AFC fluorogenic substrate (Bossy-Wetzel and Green, 1999). Whole cell lysates (50 μg protein) were incubated with 100 μM DEVD-AFC in a buffer containing 20 mM Pipes, pH 7.2, 100 mM NaCl, 1 mM EDTA, 0.1% Chaps, 10% sucrose, and 10 mM DTT at 25°C. Initial rates of substrate hydrolysis were determined using a Tecan SpectroFlu fluorimeter in the kinetic mode. Excitation was at 400 nm, and emission was at 500 nm.

Confocal microscopy

Cells were plated overnight in cell culture dishes containing a sterilized coverslip. Cells were treated with UV (90 mJ/cm²), and after a 1 h incubation the culture dishes were placed in an MS-C Temp Controller (Narishige). Images were taken every 5 min using a Nikon Eclipse TE 300 microscope and a MRC 1024ES laser scanning confocal microscope (Bio-Rad Laboratories) as described previously (Waterhouse et al., 2001). Annexin V-FITC was excited using a 488-nm line from Ar/Kr laser attenuated at 91%. Loss of plasma membrane integrity was detected by adding 0.4 μg/ml PI. Mitochondrial membranes were stained with 40 nM TMRE (Molecular Probes).

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