The role of cytochrome c in caspase activation in Drosophila melanogaster cells

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The release of cytochrome c from mitochondria is necessary for the formation of the Apaf-1 apoptosome and subsequent activation of caspase-9 in mammalian cells. However, the role of cytochrome c in caspase activation in Drosophila cells is not well understood. We demonstrate here that cytochrome c remains associated with mitochondria during apoptosis of Drosophila cells and that the initiator caspase DRONC and effector caspase DRICE are activated after various death stimuli without any significant release of cytochrome c in the cytosol. Ectopic expression of the proapoptotic Bcl-2 protein, DEBCL, also fails to show any cytochrome c release from mitochondria. A significant proportion of cellular DRONC and DRICE appears to localize near mitochondria, suggesting that an apoptosome may form in the vicinity of mitochondria in the absence of cytochrome c release. In vitro, DRONC was recruited to a >700-kD complex, similar to the mammalian apoptosome in cell extracts supplemented with cytochrome c and dATP. These results suggest that caspase activation in insects follows a more primitive mechanism that may be the precursor to the caspase activation pathways in mammals.

Introduction

Programmed cell death by apoptosis is a common way for deleting unwanted and superfluous cells (for review see Vaux and Korsmeyer, 1999). Apoptosis is mediated by a group of cysteine proteases, termed caspases (for review see Nicholson, 1999). Caspases, which cleave their substrates after an aspartate residue, are normally present as inactive precursors in cells. Upon receiving an apoptotic signal, the pro-forms (zymogens) of caspases undergo proteolytic processing to generate active enzyme (for reviews see Kumar, 1999; Kumar and Colussi, 1999; Nicholson, 1999; Wang, 2001). In mammals there are 13 caspases, some of which are involved in apoptosis, whereas others are believed to be primarily responsible for the activation and regulation of proinflammatory cytokines (Nicholson, 1999). There are a total of seven caspases in Drosophila melanogaster: DCP-1, DREDD, DRICE, DRONC, DECAY, DAMM, and STRICA (Fraser and Evan, 1997; Fraser et al., 1997; Song et al., 1997; Chen et al., 1998; Dorstyn et al., 1999a,b; Doumanis et al., 2001; Harvey et al., 2001). Of these caspases, DRONC, DREDD, DCP-1, and DRICE have been shown to play a role in the execution of cell death (Fraser and Evan, 1997; Fraser et al., 1997; Song et al., 1997; Chen et al., 1998; McCall and Stellar, 1998; Dorstyn et al., 1999a,b; Hawkins et al., 2000; for review see Kumar and Doumanis, 2000; Meier et al., 2000; Quinn et al., 2000).

DRONC is the only Drosophila caspase that contains a caspase recruitment domain, suggesting that it is the functional counterpart of CED-3 in Caenorhabditis elegans and caspase-9 in mammals (Dorstyn et al., 1999a). Genetic and gene ablation experiments have demonstrated that DRONC is essential for programmed cell death during development and is required for cell death in the fly eye, mediated by Reaper, Hid, and Grim (Hawkins et al., 2000; Meier et al., 2000; Quinn et al., 2000). DRONC interacts with and processes the effector caspase DRICE, suggesting that DRONC is an initiator caspase (Hawkins et al., 2000; Kumar and Doumanis, 2000; Meier et al., 2000). Furthermore, dronc transcript is massively up-regulated in larval salivary glands and midgut by the hormone ecdysone, which mediates programmed deletion of larval salivary glands and midgut by the hormone ecdysone, which mediates programmed deletion of larval salivary glands and midgut by the hormone ecdysone, which mediates programmed deletion of larval salivary glands and midgut by the hormone ecdysone, which mediates programmed deletion of larval salivary glands and midgut by the hormone ecdysone. Although the biochemical mechanism of DRONC activation is not well understood, genetic studies demonstrate that DARK/Dapaf-1/HAC-1, the Drosophila CED-4/Apaf-1 homologue, is required for DRONC-mediated cell death (Quinn et al., 2000), suggesting that a DARK–DRONC complex may be necessary for initial autocatalytic activation.
of DRONC. In support of this model, DRONC has been shown to interact with DARK in a caspase recruitment domain–dependent manner (Quinn et al., 2000).

In mammals, cellular stress signals lead to the release of mitochondrial cytochrome c, which binds to and enables oligomerization of the adaptor protein Apaf-1 (Li et al., 1997; Zou et al., 1997, 1999). Apaf-1, in turn, recruits the precursor form of caspase-9, an apical caspase, and thereby promotes its proximity-induced autocatalytic activation (Li et al., 1997; Zou et al., 1997, 1999). The requirement of cytochrome c in caspase activation is well documented in mammals. In mice in which the cytochrome c gene has been deleted by homologous recombination, the caspase-9–Apaf-1 pathway is severely impaired (Li et al., 2000). In contrast, in C. elegans, there is no published evidence that CED-4–mediated CED-3 activation requires cytochrome c for its oligomerization. Kanuka et al. (1999) have shown that addition of cytochrome c and dATP to Drosophila embryo extracts results in a twofold enhancement of DEVDase activity, which was not seen in extracts prepared from dapaf-1 mutant embryos. The cytochrome c/dATP–induced caspase activity was also inhibited by an ATPase inhibitor, suggesting that the ATPase activity of Dapaf-1 may be necessary for its ability to mediate caspase activation (Kanuka et al., 1999).

There are conflicting reports of cytochrome c release from the mitochondria of Drosophila cells during apoptosis. In Drosophila SL2 cells, overexpression of rpr or treatment with staurosporine or cycloheximide causes an apparent release of cytochrome c into the cytosol (Kanuka et al., 1999). On the other hand, Varkey et al. (1999) demonstrated an alteration in cytochrome c conformation, as evidenced by display of an otherwise hidden epitope, in Drosophila tissues preceding apoptosis. This alteration occurs without release of the protein into the cytosol. In cell-free studies, caspase activation was triggered by mitochondria from apoptotic cells but not by those from healthy cells. These observations suggest that in the fly, the function of cytochrome c in caspase activation may be somewhat different from its role in mammalian cells. If cytochrome c is not released from mitochondria, it is unclear if it is required for caspase activation in vivo. The analysis of endogenous Drosophila caspases has been limited due to a lack of appropriate antibodies.

In this paper, we have investigated whether cytochrome c release from mitochondria is necessary for the activation of key Drosophila apoptotic caspases, i.e., DRONC and DRICE. Using specific antibodies, we demonstrate that during apoptosis, DRONC and DRICE are rapidly processed without any cytochrome c release from mitochondria. We also show that in cell-free extracts, both DRONC and DRICE are recruited to a >700-kD complex, which is presumably required for their activation.

Results

DRONC is activated early during apoptosis of ML-DmBG2c2 (BG2) cells

For most studies reported in this paper, we have used ML-DmBG2c2 (BG2)* cells derived from the Drosophila larval central nervous system (Ui-Tei et al., 2000). The main advantage of using these cells is that, unlike the commonly used SL2 cells, BG2 cells are adherent and have a flatter morphology to allow subcellular localization studies not easily done in SL2 cells. BG2 cells undergo classical caspase-dependent apoptosis after treatment with a number of agents and serum/factor withdrawal (Ui-Tei et al., 2000). The kinetics of cell death by treatment with cycloheximide is rapid. Dying cells become morphologically distinguishable within 2–4 h and appreciable caspase activity is detectable at 4 h (Fig. 1 A), DRONC is an apical caspase in the fly, and previous studies from our laboratory, and those of others, have demonstrated that DRONC is essential for developmentally programmed cell death (Dorstyn et al., 1999a; Hawkins et al., 2000; Meier et al., 2000; Quinn et al., 2000). To test whether DRONC is activated during apoptosis of BG2 cells, we used an affinity-purified DRONC polyclonal antibody to study DRONC processing. As shown in Fig. 1 B, the 50-kD DRONC precursor was cleaved to a 36-kD (p36) fragment that was not processed any further. DRONC processing became apparent after 4 h of cycloheximide treatment of BG2, and by 16 h all proDRONC was fully cleaved to p36 fragment. Because DRONC antibody was raised against the carboxyl terminus of the protein, the processing data indicate that p36 represents the catalytic region of DRONC devoid of the prodomain and suggest that DRONC precursor is not processed into two subunits during apoptosis. We have previously noted that the recombinant prodomainless DRONC protein (p36) expressed in Escherichia coli is significantly more active than the full-length DRONC protein (p50) (Dorstyn et al., 1999a; unpublished data). Interestingly, p36 does not undergo any significant processing in E. coli. These data suggest that p36 is likely to be the active species in vivo. We also observed that DRONC is processed only to p36 species in two other Drosophila cell lines undergoing apoptosis (unpublished data). DRICE, an effector caspase, is a downstream target of DRONC. Therefore, we also analyzed DRICE processing in BG2 cells using an antibody raised against the large subunit of DRICE. The DRICE precursor was fully processed after 8 h of cycloheximide treatment, as evidenced by the disappearance of the 35-kD precursor and appearance of the 19-kD large subunit (Fig. 1 B). The withdrawal of serum and insulin from BG2 cell cultures resulted in slower death kinetics, with caspase activation only becoming apparent after 16 h of factor withdrawal (Fig. 1 D). Some processing of proDRICE was also seen around 16 and 24 h. However, full processing required longer incubation in serum-starved conditions (unpublished data).

Abbreviations used in this paper: BG2, ML-DmBG2c2; GST, glutathione-S-transferase; HA, hemagglutinin; RNAi, RNA-mediated interference; RT, room temperature.
after treatment with cycloheximide, similar to that seen in whole cell lysates. Interestingly, a significant fraction of endogenous DRONC was found in the P10 fraction that contains mitochondria, and to a lesser extent in the light membrane P100 fraction that contains various types of ER- and Golgi-derived vesicles, endosomes, etc. (Fig. 3 A). Both P10 and P100 fractions contained precursor and processed forms of DRONC. The overall level of membrane-associated DRONC protein remained unchanged after treatment with cycloheximide. Similar to DRONC, the majority of DRICE was present in the cytosolic fraction, with a small fraction associated with the heavy membrane fraction P10 (Fig. 3 A). However, DRICE was not found in the P100 fraction. Blotting of the same blots with a cytochrome c antibody showed that cytochrome c was associated with the mitochondrial fraction, and there was no apparent decrease in mitochondrial cytochrome c in cells treated with cycloheximide (Fig. 3 A), suggesting that cytochrome c was not being released from the mitochondria in cells undergoing apoptosis. Consistent with this observation, there was no cytochrome c detected in the P100 or S100 fraction.

To test whether lack of cytochrome c release during apoptosis is common to apoptosis induced by various forms of stress, we performed experiments in which BG2 cells were fractionated after withdrawal of serum and insulin for various lengths of time. As the kinetics of cell death after serum/insulin withdrawal are much slower than in cycloheximide-treated cells (Fig. 1 C), it was possible to observe subtle changes in protein levels in various fractions. As before, the majority of DRONC was present in the cytosolic fraction, but significant amounts were also associated with the P10 and P100 fractions (Fig. 3 B). Interestingly, we could see a significant increase in membrane-associated DRONC in cells exposed to serum-starved conditions for 16–24 h. Both processed and precursor forms of DRONC were found in all fractions. As shown previously (Fig. 1 D), most proDRICE remains in its precursor form 24 h after serum withdrawal and was largely found in the cytosolic fraction (Fig. 3 B). As in the case of cycloheximide-treated BG2 cells, there was no apparent release of cytochrome c from the mitochondrial fraction in cells exposed to serum-free conditions (Fig. 3 B).
Cytochrome \( c \) remains associated with mitochondria in dying BG2 cells

To confirm our cell fractionation experiments, we performed immunostaining of BG2 cells undergoing apoptosis. Staining with the mitochondrial marker MitoTracker showed punctate staining that was similar to that seen with cytochrome \( c \) staining (Fig. 4). Upon treatment with cycloheximide, there were clear morphological changes in treated cells that became evident at 4 h, however, the majority of the MitoTracker and cytochrome \( c \) staining was still punctate. These results suggest that after treatment with cycloheximide, mitochondria remain intact in dying cells and the majority of the cytochrome \( c \) remains associated with mitochondria (Fig. 4). Staining of cells with the cationic dye DiOC\(_6\) over 8 h indicated that mitochondrial membrane potential is not altered in BG2 cells after cycloheximide treatment (unpublished data). Staining of endogenous DRONC, using the affinity-purified DRONC antibody, showed that the majority of the protein is cytosolic with some punctate staining that appears to become more concentrated in the perinuclear regions of the cells treated with cycloheximide (Fig. 4). We also determined the localization of DRICE, a key downstream caspase activated by DRONC (Meier et al., 2000). We used an antibody for DRICE that detects only the processed form. As shown in Fig. 4, at 0 h, there was little or no DRICE staining in cells, but by 4 h, significant processed DRICE was detected in BG2 cells. At 8 h, considerably more active DRICE was evident, and in many cells it showed punctate staining or appeared to have accumulated in large aggregates (Fig. 4).

DRONC partly localizes in the vicinity of mitochondria in BG2 cells

To test whether DRONC colocalizes with mitochondria and cytochrome \( c \), we performed double staining of BG2 cells. In untreated cells, a significant proportion of endogenous DRONC was found to localize with mitochondria, as detected by both MitoTracker (Fig. 5 A) or cytochrome \( c \) (Fig. 5 B) costaining. Similar results were obtained when serum withdrawal was used to induce apoptosis in BG2 cells (Fig. 6 A). The kinetics of cell death due to serum withdrawal are relatively slow (Fig. 1). Nonetheless, caspase activation, as measured using the processed DRICE–specific antibody, was clearly visible (Fig. 6 B). Interestingly, some active DRICE also appeared to localize with mitochondria, cytochrome \( c \), and DRONC in serum-deprived cells even before any morphological features of apoptosis became evident (Figs. 5 C and 6 B; unpublished data).

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**Figure 3.** DRONC activation does not require cytochrome \( c \) release from mitochondria. BG2 cells treated with cycloheximide (A) or deprived of serum/insulin (B) for various lengths of time were gently lysed and fractionated by differential centrifugation to separate heavy membrane (P10, containing mitochondria), light membrane (P100), and cytosol (S100). Aliquots from each fraction were electrophoresed through 15% polyacrylamide gels and immunoblotted using an anti–cytochrome \( c \) monoclonal antibody (top), anti-DRONC polyclonal antibody (middle), or anti-DRICE polyclonal antibody (bottom). Signals were detected by ECL. The 50- and 36-kD DRONC bands represent the precursor and processed forms of DRONC, respectively.

**Figure 4.** Cytochrome \( c \) is not released from mitochondria during BG2 cell apoptosis. BG2 cells treated with cycloheximide for various durations were fixed and stained with the mitochondrial marker MitoTracker red, or immunostained with either anti–cytochrome \( c \) monoclonal antibody or polyclonal antibodies for DRONC or DRICE. After incubation with appropriate FITC-coupled secondary antibodies, cells were visualized by fluorescence microscopy. Note that the DRICE antibody used in these experiments only recognizes the processed active form of DRICE, and thus cells undergoing apoptosis show a more intense staining for DRICE than the control at 0 h.
To ensure that lack of cytochrome c release during apoptosis is not a cell- and signal-specific process, we also used l(2)mbn cells (Ress et al., 2000) undergoing apoptosis in response to ecdysone treatment. Ecdysone treatment of l(2)mbn cells results in transcriptional up-regulation of dronc expression, followed by accumulation and processing of proDRONC to p36 (unpublished data). RNAi experiments have shown that DRONC up-regulation is essential for ecdysone-mediated apoptosis of l(2)mbn cells (unpublished data). Consistent with the results in BG2 cells, both cell fractionation and immunocytochemistry experiments suggested that in l(2)mbn cells undergoing ecdysone-mediated apoptosis, caspase activation occurred without any significant release of cytochrome c (Fig. 7; unpublished data).

**DEBCL overexpression fails to cause cytochrome c release**

In mammals, members of the proapoptotic Bax subfamily of proteins translocate from the cytosol to the mitochondrial membrane and cause a release of cytochrome c during apoptosis (Gross et al., 1999; Kluck et al., 1999; for review see Strasser et al., 2000; Wei et al., 2000). Recent studies have shown that Bax and Bak coalesce into large clusters adjacent to mitochondria (Nechushtan et al., 2001). In *Drosophila*, there are two Bax-like proteins, DEBCL (also known as Dro-b-1, dBorg, or dBok) and BUFFY (Brachmann et al., 2000; Colussi et al., 2000; Igaki et al., 2000; Zhang et al., 2000). Only DEBCL has been shown to be proapoptotic (Brachmann et al., 2000; Colussi et al., 2000; Igaki et al., 2000; Zhang et al., 2000). Overexpression of DEBCL in transfected mammalian and *Drosophila* SL2 cells results in its localization to mitochondria (Igaki et al., 2000; Zhang et al., 2000). Overexpression of DEBCL in mammalian cells has been shown to cause release of cytochrome c, suggesting that DEBCL is functionally similar to Bax (Zhang et al., 2000). To test whether DEBCL can induce release of cytochrome c in insect cells, we transfected a hemagglutinin (HA)-tagged DEBCL expression construct into BG2 cells. As shown in Fig. 8 A, in transfected cells, DEBCL expression corresponds to the pattern of mitochondria, as assessed by colocalization of DEBCL with cytochrome c. However, there was no apparent release of cytochrome c in DEBCL-transfected cells (Fig. 8, A–C). Interestingly, in transfected
Figure 7. Lack of cytochrome c release during ecdysone-induced caspase activation in l(2)mtn cells. Drosophila l(2)mtn cells were treated with ecdysone for 24 h. After this, cells were either immunostained for active DRICE and cytochrome c (A) or fractionated (B) as in Fig. 3. After 24 h of ecdysone treatment, >80% of the cells were morphologically apoptotic and the majority of DRONC was processed into p36 (unpublished data). (A) Immunostaining with the active DRICE antibody clearly showed that cells contained processed DRICE. Please note that in B, the small amount of cytochrome c present in all cytosolic lanes is unlikely to represent cytochrome c released from mitochondria in cells undergoing apoptosis, as it does not show an increase over time. Furthermore, there was no reduction in the mitochondrial cytochrome c seen over the course of ecdysone treatment of cells.

Figure 8. Ectopic expression of DEBCL does not induce cytochrome c release. BG2 cells were transfected with a DEBCL–HA expression construct. 24 h after transfection, cells were immunostained with an anti-HA monoclonal antibody and with either cytochrome c monoclonal antibody (A), the DRONC polyclonal antibody (B), or the active DRICE antibody (C). After incubation with FITC–DEBCL–HA or rhodamine–cytochrome c and DRONC coupled secondary antibodies, cells were visualized by fluorescence microscopy. The column on the right shows merged images of the DEBCL and cytochrome c costaining (A), DEBCL and DRONC costaining (B), or DEBCL and active DRICE costaining (C). In C, note that the DEBCL-expressing cell is positive for active DRICE. (D) Extracts prepared from DEBCL–HA-transfected BG2 cells were fractionated by centrifugation into heavy membranes (containing mitochondria), light membranes, and cytosol. Proteins were separated through 15% polyacrylamide gels and immunoblotted with an anti-HA monoclonal antibody (to detect DEBCL expression; top), anti-cytochrome c monoclonal antibody (middle), and anti-DRONC polyclonal antibody (bottom).

Figure 9. DRONC forms a large complex in response to cytochrome c and dATP

In mammalian cell extracts, addition of cytochrome c and dATP results in the formation of an ~700-kD complex, commonly known as an apoptosome (Cain et al., 1999, 2000). Studies using purified components have demonstrated that the apoptosome, consisting of Apaf-1, cytochrome c, and procaspase-9, is necessary for caspase-9 activation (Li et al., 1997; Cain et al., 1999; Rodriguez and Lazebnik, 1999; Zou et al., 1999). As formation of an apoptosome in Drosophila has not been demonstrated and because cytochrome c is not released from mitochondria during apoptosis, we tested whether a cytochrome c–dependent apoptosome containing DRONC is formed in Drosophila cells. Cell extracts prepared from BG2 cells were fractionated by gel filtration chromatography and individual fractions were analyzed by immunoblotting using specific antibodies. In cell extracts kept at 4°C, the majority of DRONC was eluted in its monomeric form (50 kD) in fractions 20–22 (Fig. 9). Extracts that were incubated at 27°C with or without cytochrome c and dATP showed a shift of some of the DRONC protein to fractions 3–5, which correspond to a molecular mass of >670 kD (Fig. 9 A). The shift in the absence of added cytochrome c may suggest that endogenous...
cytochrome c present in cell extracts could be sufficient to allow the formation of the large complex containing DRONC (see below). We have seen similar results using mammalian cell extracts (unpublished data). Drosophila cells grow at 27°C, however, when the cell extracts were incubated at 37°C, the majority of the DRONC was recruited to the >700-kD complex and there was increased processing of proDRONC and proDRICE. The reason for this is not clear, however we have noticed that recombinant DRONC and DRICE and extracts prepared from apoptotic BG2 cells show considerably more caspase activities at 37°C than at 27°C (unpublished data).

We also determined whether the large complex contains DRICE. In cell extracts incubated at 4°C, the majority of the DRICE precursor remained in its monomeric form, although some appeared to be dimeric (Fig. 9 B). Incubation of cell extracts at 27°C or 37°C, with or without cytochrome c/dATP, resulted in the recruitment of a fraction of DRICE to the high molecular mass complex (Fig. 9 B). Interestingly, in extracts incubated at 37°C, most of the DRICE in the high molecular mass complex was processed, whereas most of the monomeric DRICE was in the precursor form. These results suggest the formation of an apoptosome containing DRONC and DRICE in Drosophila cell extracts.

To further explore the role of cytochrome c in the formation of the DRONC-containing complex, we immunodepleted cytochrome c from S100 fractions (Fig. 10 A). These fractions were then subjected to gel filtration experiments. When incubated at 27°C, a small fraction of DRONC was found in the high molecular mass complex (Fig. 10 B). Addition of cytochrome c and dATP caused a significant increase
in the recruitment of DRONC to the >700-kD complex. Immunoblotting the fractions with the cytochrome c antibody showed that incubation of S100 at 27°C results in the recruitment of a significant proportion of cytochrome c to the >700-kD complex (Fig. 10 C). Interestingly, only dimeric (26 kD) cytochrome c was detected in the >700-kD complex. These results suggest that cytochrome c and dATP, at least in part, are responsible for the formation of the complex.

**Discussion**

Release of cytochrome c from mitochondria is one of the key steps in mediating caspase activation in mammalian cells (for reviews see Green and Reed, 1998; Wang, 2001). However, consistent with a previous report (Varkey et al., 1999), we do not find any cytochrome c in cytosolic fractions of Drosophila cells undergoing apoptosis. Importantly, the novel finding reported in this paper is that endogenous DRONC activation can occur during apoptosis without any apparent cytochrome c in the cytosolic fraction, suggesting that cytochrome c release from mitochondria is not required for DRONC activation. Similarly, DRICE, the downstream target of DRONC, was also activated in the absence of cytosolic cytochrome c, further confirming that caspase activation in Drosophila cells occurs without the release of cytochrome c from mitochondria.

Treatment of cells with multiple death stimuli, including cycloheximide, factor withdrawal, edcsyne, or transfection of the Bax-like protein DEBCL, failed to induce the release of detectable cytochrome c. Our immunolocalization studies suggest that cytochrome c remained associated with mitochondria in cells undergoing apoptosis. Moreover, the mitochondria appeared remarkably intact even in late stage apoptotic cells. However, in some cells undergoing apoptosis there appeared to be an accumulation of mitochondria in the perinuclear region of the cells, which is similar to that reported in mammalian cells expressing truncated Bid (Li et al., 1998). Interestingly, a fraction of endogenous DRONC colocalized with mitochondria. Cell fractionation studies suggest that a significant proportion of the DRONC precursor and processed DRONC is associated with both the mitochondrial membrane fraction and the light membrane fraction. Therefore, it is possible that membrane recruitment of DRONC is important for its activation. Overexpression of DEBCL results in its localization to mitochondria (Igaki et al., 2000; Zhang et al., 2000). Again, in the DEBCL-overexpressing cells, a fraction of DRONC appeared to colocalize with mitochondria and DEBCL, suggesting that translocation of DRONC may be important for caspase activation.

If cytochrome c is not released from mitochondria during apoptosis, how may caspase activation occur in Drosophila cells? Previous work using cytochrome c monocholin antibodies has shown that in Drosophila egg chambers and in SL2 cells, cytochrome c undergoes conformational changes within the mitochondria before the onset of apoptosis (Varkey et al., 1999). However, cytochrome c was not released from mitochondria during apoptosis, as we have found in our studies with BG2 and l(2)mbn cells. This is in contrast to apoptosis in mammalian cells where cytochrome c release from mitochondria is rapid and complete (Green and Reed, 1998; Goldstein et al., 2000). The work by Varkey et al. (1999) also demonstrates that mitochondria isolated from apoptotic SL2 cells can mediate caspase activation in vitro. These results suggest that although both mitochondria and cytochrome c play a role in apoptosis, in Drosophila, cytochrome c release from mitochondria does not occur. One possibility is that cytochrome c is released but remains tethered to the mitochondrial membrane (Varkey et al., 1999). In such a case, an apoptosome, albeit somewhat different from the mammalian apoptosome, may form in the vicinity of mitochondria. Our observations that in BG2 cells undergoing apoptosis, mitochondria become somewhat clustered and a significant proportion of DRONC colocalizes with mitochondria support this possibility. Because proapoptotic proteins such as DEBCL and Hid also localize to mitochondria, mitochondria clearly play a role in apoptosis in Drosophila.

We explored the possibility of apoptosome formation by gel filtration of BG2 cell extracts and testing for recruitment of DRONC to high molecular mass complexes. Incubation of BG2 cell extracts containing endogenous cytochrome c at 27°C–37°C resulted in the recruitment of cytosolic DRONC to a complex of >700 kD. Furthermore, a large fraction of DRONC in the complex was in processed form, suggesting that recruitment to the putative apoptosome may be necessary for DRONC activation. Interestingly, a significant proportion of DRICE was also found in the >700-kD complex, most of which was fully processed when extracts were incubated at 37°C. As DRICE has been shown to directly interact with DRONC, the recruitment of DRICE may be required for the initial DRONC-mediated activation of DRICE. This prediction is consistent with the studies with caspase-9/Apaf-1 apoptosome, which also mediates the initial activation of caspase-3 (Bratton et al., 2001).

Given that cytochrome c is not released from mitochondria in Drosophila cells, yet cytochrome c addition to Drosophila cell extracts can enhance the formation of an apoptosome-like complex and caspase activation, it is reasonable to hypothesize that caspase activation in insects follows a more primitive mechanism, which may be the precursor to the caspase activation pathways in mammals. However, our data presented in this paper does not rule out the possibility that a factor(s) other than cytochrome c may be involved in caspase activation in Drosophila cells. In future studies, the elucidation of the exact nature of the Drosophila apoptosome may shed light on the mechanism of apoptosome formation and caspase activation.

**Materials and methods**

**Cell culture**

*Drosophila* BG2 cells derived from larval central nervous system (Ui-Tei et al., 2000) were obtained from K. Ui-Tei (Nippon Medical School, Tokyo, Japan). Cells were maintained in Schneider’s cell medium (GIBCO BRL) supplemented with a 0.5% penicillin-streptomycin mix (GIBCO BRL), 10% heat-inactivated FBS, 1% glutamine (GIBCO BRL), and 10 μg/ml insulin (Novartis). Cells were cultured at a density of 10^4 cells/ml at 27°C. l(2)mbn cells (gift from A. Dorn, Johannes Gutenberg University, Mainz, Germany; Ress et al., 2000) were also grown in Schneider’s medium supplemented with 10% FBS. Cells, 1.5 × 10^6/well, were seeded in six-well plates and treated with edcsyne (10 μM; Sigma) for the desired time.
Cell death and caspase processing assays

5 × 10^6 cells were washed in PBS and incubated with 20 μg/ml cycloheximide (Sigma-Aldrich) or were grown in medium depleted of FBS and insulin. Cells were harvested at various time points after apoptotic induction and the number of surviving cells was estimated by trypan blue exclusion. For immunoblots with caspase antibodies, cells were centrifuged at 400 g for 5 min and pellets were resuspended in 50 μl PBS, mixed with an equal volume of 2× SDS protein loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol), and then boiled for 10 min before SDS-PAGE.

Caspase assays

Cells were harvested and resuspended in 100 μl caspase assay buffer (0.1 M Hepes, pH 7, 0.1% CHAPS, 10% FBS, 10 mM DTT, supplemented with 1× protease inhibitor cocktail; Roche Biochemicals). Cells were lysed by three cycles of freeze–thawing. Cell debris was centrifuged at 9,000 g for 10 min at 4°C. The cleared lysates (100 μg) were assessed for caspase activity by incubation with 100 μM of either DEVD-7-amino-4-methylcoumarin (DEVD-avc; Enzyme System Products) or VDAD-avc (California Peptide Research) in a total volume of 40 μl caspase assay buffer at 37°C for 30 min. We also tested caspase activity at 27°C because Drosophila cells grow at this temperature. However at 27°C, measured caspase activity was considerably lower. Reactions were transferred to acyl cuvettes (Sarstedt), and 3 ml water was added before analysis on a luminiscence spectrometer (excitation 385 nm, emission 460 nm; PerkinElmer).

RNA interference

Regions of cDNA for dronc (nt 781–1047), dark (nt 3603–3962), and the negative control mouse N4WBP5 (~700-bp coding region; Harvey et al., 2002) were PCR amplified using appropriate primers, and cloned into pGEM-T Easy (Promega). Plasmids were linearized and RNA synthesized using T7 and SP6 Megascript kits (Ambion). Sense and antisense strands were annealed to generate dsRNA, and RNA quality was analyzed on agarose gel. dsRNA (27 μM) was added to cells in 1 ml serum-free media and mixed vigorously. Cells were incubated for 1 h followed by the addition of 2 ml of media supplemented with 10% FBS. Cells were incubated overnight and then treated with cycloheximide for 6 h before cell lysis and immunoblotting.

Cell fractionation

5 × 10^6 cells were harvested by centrifugation at 400 g for 5 min. Cell pellets were resuspended in 200 μl of lysis buffer A (20 mM Hepes-KOH, pH 7.5, 50 mM KCl, 1 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT in 250 mM sucrose and supplemented with protease inhibitor cocktail; Roche Biochemicals), and incubated on ice for 5 min. Cells were homogenized by 10 strokes with a tight fitting pestle, and homogenates were centrifuged at 10,000 g for 20 min at 4°C. Pellets, consisting of unbroken cells and nuclei, were resuspended and resuspended in 100 μl PBS. The supernatants were centrifuged at 10,000 g for 15 min at 4°C, and the heavy membrane/mitochondrial pellets were washed in cold buffer, resuspended in 30 μl lysis buffer A, and stored at −70°C. Supernatants were further centrifuged at 100,000 g for 30 min at 4°C. The resulting pellets (P100) consisting of light membrane fractions were resuspended in 30 μl lysis buffer A and stored at −70°C. Resulting supernatants (S100) were also stored at −70°C. For protein gel analysis, 4× SDS protein loading buffer was added to each fraction and samples were resolved on 8–15% SDS-PAGE gels.

Affinity purification of DRONC antibodies

Anti-DRONC rabbit polyclonal sera was passed through a column of glutathione-agarose (GST) coupled to cytochrome c–depleted extracts to remove GST-specific antibodies. Flowthrough from this step was affinity purified against DRONC p14–GST (Quinn et al., 2000) coupled to cytochrome c–depleted Sepharose 4. Bound proteins were eluted into Tris-HCl (pH 8.6) with 100 mM glycine (pH 2.5) and dialyzed against PBS. Affinity-purified antibodies were diluted in 50% glycerol and stored at −20°C.

Generation and affinity purification of anti-active DRICE antibodies

Active DRICE–specific antibodies used in immunofluorescence studies (Figs. 4–8) were raised in rabbits using a synthetic octapeptide corresponding to the COOH terminus of the DRICE large subunit (QRSQETD) conjugated with keyhole limpet hemocyanin as the immunogen (Covance). Active DRICE–specific antibodies were purified by sequential protein affinity purification. Antisera were first applied to an affinity column containing full-length DRICE with an inactivating active site mutation (C211A). The flowthrough from this column was applied to an affinity column containing the DRICE large subunit ending at the caspase cleavage site TETD (1–230). Bound antibody was eluted using 100 mM glycine, pH 2.5. Immunoblotting of the eluted anti-DRICE antibodies showed that they were specific for the cleaved large subunit of active DRICE. They did not detect full-length DRICE or other caspases, including the closely related DCP-1 (unpublished data).

A second rabbit anti-DRICE polyclonal antibody, used for immunoblotting, was generated using a version of DRICE that extends from residue 80 to the COOH terminus, followed by a COOH-terminal 6× His tag as the immunogen. DRICE-reactive antibodies were purified by binding these antibodies to the antigen and washing extensively. DRICE-reactive antibodies were eluted as above for the DRICE cleavage-specific antibodies.

Immunoblotting

After SDS-PAGE, protein was transferred to a PVDF membrane (Dupont). The membranes were blocked in 5% skim milk (Diploma)–PBS/0.05% Tween 20 solution overnight at 4°C. Blots were incubated with 0.5 μg/ml anti-cytochrome c mouse antibody 76H2.C12 (BD PharmMingen), 1.5 μg/ml affinity-purified anti-DRONC rabbit antibody, 0.1 μg/ml anti-BA mouse antibody (Roche Biochemicals), or 1.5 μg/ml anti-DRICE rabbit antibody, followed by incubation with the corresponding HRP-conjugated secondary antibody. Signals were visualized by ECL (Amershams Pharmaei Biotech).

Immunofluorescence

2 × 10^6 cells were grown on coverslips in 35-mm dishes. After apoptotic induction, cells were washed in PBS. For MitoTracker staining, cells were incubated with 300 nM MitoTracker red (Molecular Probes) in PBS with 1% FBS for 15 min at room temperature (RT). Coverslips were either mounted on slides in antifade solution (80% glycerol, 1% propylgallate) or were fixed with 3% paraformaldehyde for 10 min at RT, washed in PBS, and then permeabilized in 0.1% Triton X-100 for 10 min at RT before antibody staining. Cells were treated with anti-cytochrome c mouse antibody clone 6H2.B4 (BD Pharmingen) at 2.5 μg/ml, affinity-purified anti-DRONC rabbit antibody at 4 μg/ml, and/or anti-BA high affinity rabbit antibody (Roche Biochemicals) at 1 μg/ml and anti-active DRICE antibody at 4 μg/ml. All antibodies were diluted in PBS (0.1% FBS) and incubated with cells overnight at 4°C. Cells were then washed three times in PBS and incubated with FITC-conjugated anti-mouse, –rabbit, or –rat secondary antibodies and rhodamine-conjugated anti-rabbit or –mouse antibodies for 1 h at RT. Cells were again washed several times in PBS and coverslips were mounted onto slides in antifade solution. Cell staining was visualized under fluorescent microscopy.

Cell filtration chromatography

10^5 BG2 cells were resuspended in 200 μl buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT supplemented with protease inhibitor cocktail; Zou et al., 1999) and lysed by freeze–thaw. Cell debris was pelleted twice by centrifugation at 10,000 g for 20 min at 4°C. Supernatants were further centrifuged at 100,000 g for 30 min at 4°C. Resulting lysates (S100) were incubated at 4°C, 27°C, or 37°C alone or in the presence of 2 μg cytochrome c and 2 mM dATP. Proteins were then fractionated through a Superdex 200 column (Amershams Pharmacia Biotech). Fractions were collected 18 min after sample injection and a total of 42 fractions were collected at a rate of 0.4 ml/fraction/min. Aliquots from each fraction were resolved by electrophoresis through 8–15% polyacrylamide gels. For immunodepletion of cytochrome c, S100 lysates were incubated overnight at 4°C with an anti-cytochrome c antibody 6H2 (BD PharmMingen). Antigen–antibody complexes were removed by protein G–Sepharose, and cytochrome c–depleted extracts were fractionated through a Superdex 200 column after incubation at 27°C alone or in the presence of 2 μg cytochrome c and 2 mM dATP.

We are grateful to Dr. K. Ui-Tei for BG2 cells, Dr. A. Dom for l(2)mbn cells, and members of our laboratory for sharing ideas and reagents.

This work was supported by a grant and a fellowship to S. Kumar from the National Health and Medical Research Council of Australia.

Submitted: 29 November 2001
Revised: 1 February 2002
Accepted: 4 February 2002

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References

Baebreckc, E.H. 2000. Steroid regulation of programmed cell death during Drosophila development. *Cell Death Differ.* 7:1057–1062.

Brachmann, C.B., O.W. Jassim, B.D. Wachsmuth, and R.L. Cagan. 2000. The Drosophila bcl-2 family member db1org-1 functions in the apoptotic response to UV-irradiation. *Curr. Biol.* 10:547–550.

Bratton, S.B., G. Walker, S.M. Srivinava, X.M. Sun, M. Butterworth, E.S. Alnemri, and G.M. Cohen. 2001. Recruitment, activation and retention of caspases-9 and -3 by Apaf-1 apotosome and associated XIAP complexes. *EMBO J.* 20:998–1009.

Cain, K., D.G. Brown, C. Langlais, and G.M. Cohen. 1999. Caspase activation involves the formation of the aposome, a large (~700 kDa) caspase-activating complex. *J. Biol. Chem.* 274:22686–22692.

Cain, K., S.B. Bratton, C. Langlais, G. Walker, D.G. Brown, X.-M. Sun, and G.M. Cohen. 2000. Apaf-1 oligomerises into biologically active ~700-kDa and inactive ~1.4MDa apotosome complexes. *J. Biol. Chem.* 275:6067–6070.

Chen, P., A. Rodriguez, R. Erskine, T. Thach, and J.M. Abrams. 1998. Dredd, a novel effector of the apoptosis activators reaper, grim, and hid in Drosophila. *Dev. Biol.* 201:202–216.

Colussi, P.A., L.M. Quinn, D.C.S. Huang, M. Coombe, S.H. Read, H. Richardson, and S. Kumar. 2000. DEBCL, a proapoptotic Bcl-2 homologue, is a component of the Drosophila melanogaster cell death machinery. *J. Cell Biol.* 148:793–802.

Dorstyn, L., P.A. Colussi, L.M. Quinn, H. Richardson, and S. Kumar. 1999a. DRONC, an edcysone-inducible Drosophila caspase. *Proc. Natl. Acad. Sci. USA.* 96:4307–4312.

Dorstyn, L., S.H. Read, L.M. Quinn, H. Richardson, and S. Kumar. 1999b. DECA, a novel Drosophila caspase related to mammalian caspase-3 and caspase-7. *J. Biol. Chem.* 274:30778–30783.

Doumanis, J., L.M. Quinn, H. Richardson, and S. Kumar. 2001. STRICA, a novel Drosophila melanogaster caspase with an unusual active/cysteine-rich pro-domain, interacts with Diap1 and Diap2. *Cell Death Differ.* 8:387–394.

Fraser, A.G., and G.I. Evan. 1997. Identification of a Drosophila melanogaster ICE/CED-3-related protease, d ICE. *EMBO J.* 16:2805–2813.

Fraser, A.G., N.J. McCarthy, and G.I. Evan. 1997. D rICE is an essential caspase required for apoptotic activity in Drosophila cells. *EMBO J.* 16:6192–6199.

Goldstein, J.C., N.J. Waterhouse, P. Jiun, G.I. Evan, and D.R. Green. 2000. The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nat. Cell Biol.* 2:156–162.

Green, D.R., and J.C. Reed. 1998. Mitochondrion and apoptosis. *Science.* 281:1309–1312.

Gross, A., X.M. Yin, K. Wang, M.C. Wei, J. Jockel, C. Milliman, H. Erdjument-Bromage, P. Tempst, and S.J. Korsmeyer. 1999. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL protects mitochondria from cytochrome c release. *J. Biol. Chem.* 274:1156–1163.

Harvey, K.F., L.M. Shearwin-Whitty, A. Fotia, R.G. Parson, and S. Kumar. 2002. N/AWPB5, a potential target for ubiquitination by the Nedd4 family of proteins, is a novel Golgi-associated protein. *J. Biol. Chem.* In press.

Harvey, N.J., T. Daish, K. Mills, L. Dorstyn, L.M. Quinn, S.H. Read, H. Richardson, and S. Kumar. 2001. Characterisation of the Drosophila caspase, DMM. *J. Biol. Chem.* 276:25342–25350.

Hawkins, C.J., S.J. Leevers, and G.I. Evan. 2000. Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. *Cell.* 101:389–399.

Li, P., D. Nijhawan, I. Budhiahdaro, S.M. Srivinava, M. Ahmad, E.S. Alnemri, and X. Wang. 1997. Cytochrome c and DAPTP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell.* 91:479–489.

McCall, K., and H. Steller. 1998. Requirement for DCP-1 caspase during Drosophila oogenesis. *Science.* 279:230–234.

Meier, P., J. Silke, S.J. Leevers, and G.I. Evan. 2000. The Drosophila caspase DRONC is regulated by Diap1. *EMBO J.* 19:598–611.

Nechustan, A., C.L. Smith, I. Lammensdorf, S.-H. Yoon, and R.J. Youle. 2001. Bax and Bax coalesce into novel mitochondria-associated clusters during apoptosis. *J. Cell Biol.* 153:1265–1276.

Nicholson, D.W. 1999. Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ.* 6:1028–1042.

Quinn, L.M., L. Dorstyn, K. Mills, P.A. Colussi, P. Chen, M. Coombe, J.M. Abrams, S. Kumar, and H. Richardson. 2000. An essential role for the caspase DRONC in developmentally programmed cell death in Drosophila. *J. Biol. Chem.* 275:40416–40424.

Ress, C., M. Holtmann, U. Maas, J. Sofsky, and A. Dorn. 2000. 20-Hydroxy-ecdysone-induced differentiation and apoptosis in the Drosophila cell line, l2zimb. *Tissue Cell.* 32:464–477.

Rodrigues, A., H. Oliver, H. Zou, P. Chen, X. Wang, and J.M. Abrams. 1999. DARK is a Drosophila homolog of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. *Nat. Cell Biol.* 2:272–279.

Rodrigues, J., and Y. Laeznik. 1999. Caspase-9 and Apaf-1 form an active holoenzyme. *Genes Dev.* 13:3179–3184.

Song, Z., K. McCall, and H. Steller. 1997. DCP-1, a Drosophila cell death protease essential for development. *Science.* 273:536–540.

Strasser, A., L. O’Connor, and V.M. Dixit. 2000. Apoptosis signalling. *Annu. Rev. Biochem.* 69:217–245.

Ui-Tei, K., M. Nagano, S. Sato, and Y. Miyata. 2000. Calmodulin-dependent and independent apoptosis in cells of a Drosophila neuronal cell line. *Apoptosis.* 5:133–140.

Varkey, J., P. Chen, R. Jemmerson, and J.M. Abrams. 1999. Altered cytochrome c display precedes apoptotic cell death in Drosophila. *J. Cell Biol.* 144:701–710.

Vaux, D.L., and S.J. Korsmeyer. 1999. Cell death in development. *Cell.* 96:245–254.

Wang, X. 2001. The expanding role of mitochondria in apoptosis. *Genes Dev.* 15:2922–2933.

Wei, M.C., T. Lindest, V.K. Moonha, S. Weiler, A. Gross, M. Achiya, C.B. Thompson, and S.J. Korsmeyer. 2000. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev.* 14:2060–2071.

Zhang, H., Q. Huang, N. Ke, S. Matsuyama, B. Hammock, A. Godzik, and J.C. Reed. 2000. Drosophila pro-apoptotic Bcl-2/Bax homologue reveals evolutionarily conserved cell death mechanisms. *J. Biol. Chem.* 275:27303–27306.

Zhou, L., Z. Song, J. Tintel, and H. Steller. 1999. HAC-1, a Drosophila homolog of Apaf-1 and CED-4, functions in developmental and radiation-induced apoptosis. * Mol. Cell.* 4:745–755.

Zou, H., W.J. Henzel, X. Liu, A. Lutschg, and X. Wang. 1997. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell.* 90:405–413.

Zou, H., Y. Li, X. Liu, and X. Wang. 1999. An Apaf-1 cytochrome c multimeric complex is a functional apotosome that activates prosaposin-9. *J. Biol. Chem.* 274:11549–11556.