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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; 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 tissues during larval/pupal metamorphosis (Dorstyn et al., 1999a,b; Baehrecke, 2000). 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

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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*, suggesting that cytochrome *c* function in apoptosis may have evolved later in more complex organisms.

The role of cytochrome *c* in *Drosophila* caspase activation is not well established. Structurally, DARK is more similar to its mammalian counterpart Apaf-1 than to CED-4, in that it contains several WD40 repeats that are not found in CED-4, and it binds cytochrome *c* in vitro (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). However, it is unclear whether the *Drosophila* protein 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 C). Concomitant with caspase activity, detectable DRONC processing was also seen 16 h after serum 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 (un-

*Abbreviations used in this paper: BG2, ML-DmBG2c2; GST, glutathione-*S*-transferase; HA, hemagglutinin; RNAi, RNA-mediated interference; RT, room temperature.

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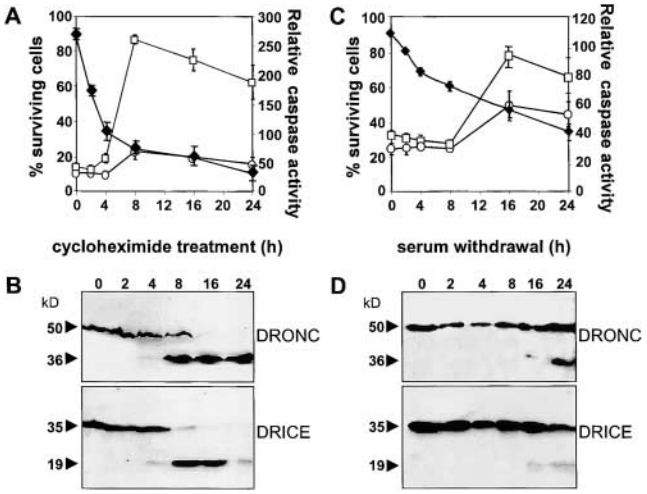

Figure 1. **Caspase activation during apoptosis of BG2 cells.** BG2 cells were treated with cycloheximide (A and B) or deprived of serum and insulin (C and D) over 24 h. (A and C) At various times after apoptotic stimuli, cells were assayed for viability by trypan blue exclusion (◆) and for caspase activity on either DEVD-amc (□) or VDVAD-amc (○). To determine caspase processing, lysates from cycloheximide-treated (B) or serum- and/or insulin-starved (D) BG2 cells were immunoblotted with anti-DRONC polyclonal antibody (top) or with an anti-DRICE polyclonal antibody (bottom) and proteins were visualized by enhanced chemiluminescence.

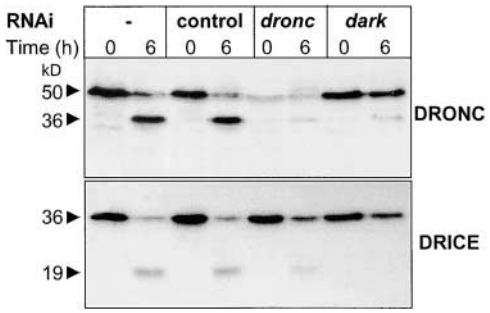

Figure 2. **DARK is required for caspase activation in BG2 cells during apoptosis.** BG2 cells were exposed to 37 nM of double stranded RNA for *dronc, dark,* or a negative control. Cells were either left untreated or treated with cycloheximide for 6 h. Cell lysates were immunoblotted using anti-DRONC and anti-DRICE antibodies.

published data). These data indicate that activation of the endogenous apical caspase DRONC and effector caspase DRICE is associated with apoptosis in BG2 cells.

Genetic studies show that DARK is required for the proapoptotic function of DRONC (Quinn et al., 2000). To test whether DARK is necessary for caspase activation in BG2 cells, we used double-stranded RNA–mediated interference (RNAi) to ablate endogenous *dark* transcript. Northern blot analysis suggested that *dark* RNAi caused a significant reduction in *dark* mRNA in BG2 cells (unpublished data). Exposure of BG2 cells with cycloheximide in the presence of *dark* RNAi resulted in an inhibition of DRONC and DRICE processing, suggesting that DARK is necessary for caspase activation (Fig. 2). We also used *dronc* RNAi to test whether reduction in the levels of endogenous DRONC protein would affect DRICE processing. *dronc* RNAi significantly reduced endogenous DRONC protein in BG2 cells and resulted in an inhibition of DRICE processing in cells undergoing apoptosis (Fig. 2). These results are consistent with DRONC being an initiator caspase that mediates DRICE activation during apoptosis.

DRONC and DRICE are activated without any apparent release of mitochondrial cytochrome *c*

To analyze the role of cytochrome *c* in DRONC activation, we exposed BG2 cells to cycloheximide or serum withdrawal for various lengths of time and subjected them to cell fractionation. Cell fractions were then analyzed by immunoblotting using cytochrome *c* and DRONC antibodies. As shown in Fig. 3 A, the majority of DRONC was present in the S100 (cytosolic) fraction and showed substantial processing 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).

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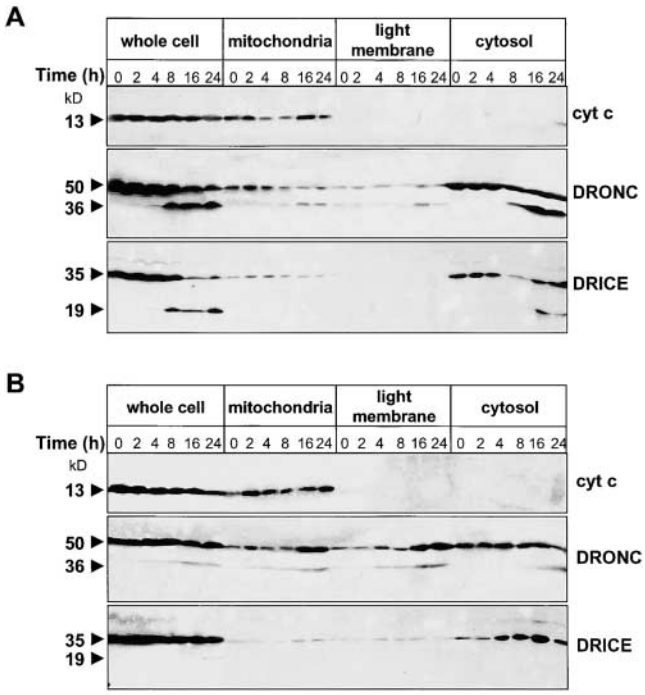

Figure 3. **DRONC activation does not require cytochrome *c* release from mitochondria.** BG2 cells treated with cycloheximide (A) or deprived of serum/insulin (B) for various lengths of time were gently lysed and fractionated by differential centrifugation to separate heavy membrane (P10, containing 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.

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).

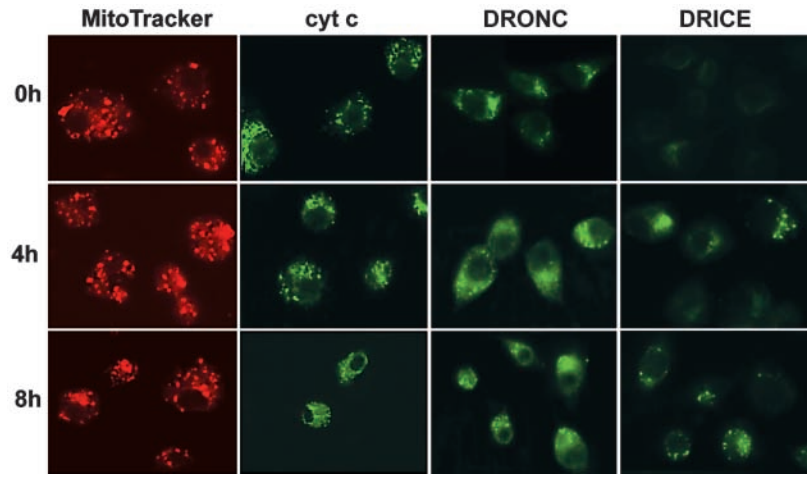

Figure 4. **Cytochrome *c* is not released from mitochondria during BG2 cell apoptosis.** BG2 cells treated with cycloheximide for various durations were fixed and stained with the mitochondrial marker MitoTracker red, or immunostained with either anti–cytochrome *c* monoclonal antibody or polyclonal 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.

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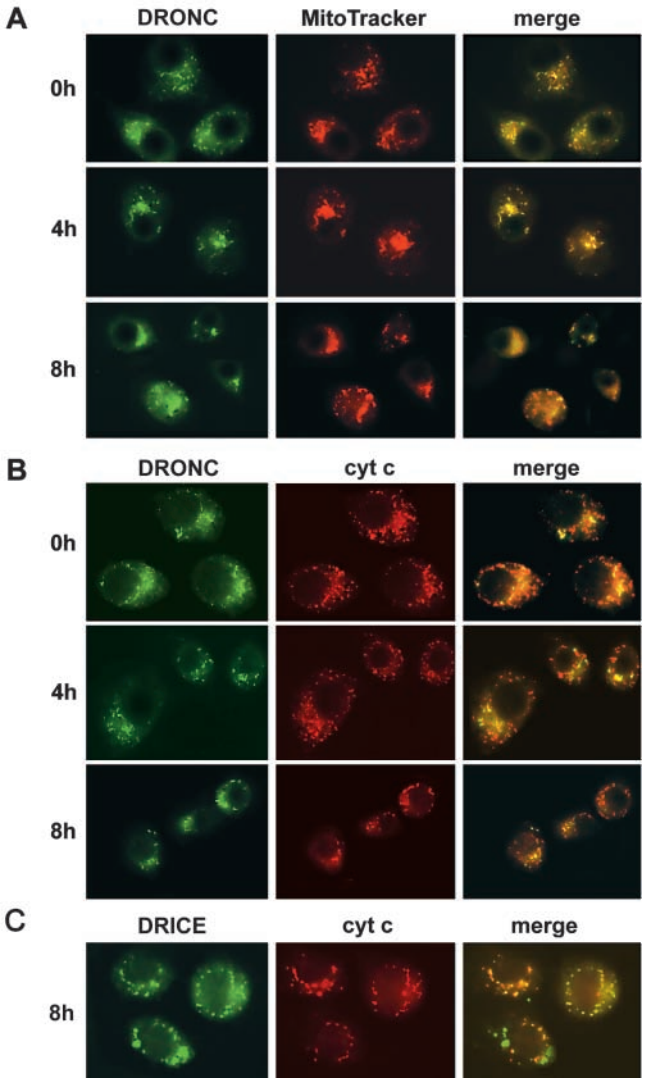

Figure 5. **DRONC and active DRICE partially colocalize with mitochondria in BG2 cells.** BG2 cells treated with cycloheximide for various lengths of time were costained with anti-DRONC polyclonal antibody and either MitoTracker red (A) or anti–cytochrome *c* monoclonal antibody (B). After incubation with FITC- (DRONC) or rhodamine- (cytochrome *c*) coupled secondary antibodies, cells were visualized by fluorescence microscopy using appropriate filters. The last column depicts merged images of the DRONC and MitoTracker costaining (A) or DRONC and cytochrome *c* costaining (B). (C) BG2 cells treated with cycloheximide for 8 h were double stained for active DRICE (FITC) and cytochrome *c* (rhodamine).

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

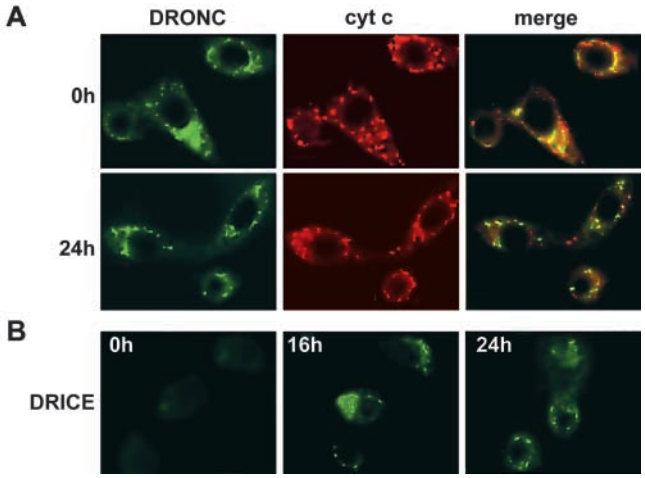

Figure 6. **Cytochrome *c* localization in BG2 cells is not altered after serum deprivation.** (A) Untreated BG2 cells or those deprived of serum and insulin for 24 h were fixed and costained with anti-DRONC polyclonal antibody and anti–cytochrome *c* monoclonal antibody. After incubation with FITC- (DRONC) or rhodamine- (cytochrome *c*) coupled secondary antibodies, cells were visualized by fluorescence microscopy using appropriate filters. The last column depicts merged images of the DRONC and cytochrome *c* costaining. (B) BG2 cells deprived of serum and insulin for various lengths of time were fixed and immunostained with anti-DRICE polyclonal antibody. As the anti-DRICE antibody only detects the active processed form of DRICE, immunofluorescence is seen in apoptotic cells only.

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 Drob-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

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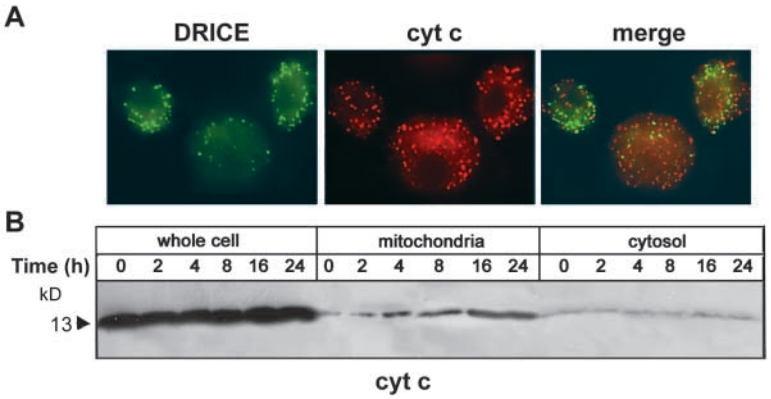

Figure 7. **Lack of cytochrome *c* release during ecdysone-induced caspase activation in *l(2)mbn* cells.** *Drosophila l(2)mbn* cells were treated with ecdysone for 24 h. After this, cells were either immunostained for active DRICE and cytochrome *c* (A) or fractionated (B) as in Fig. 3. After 24 h 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.

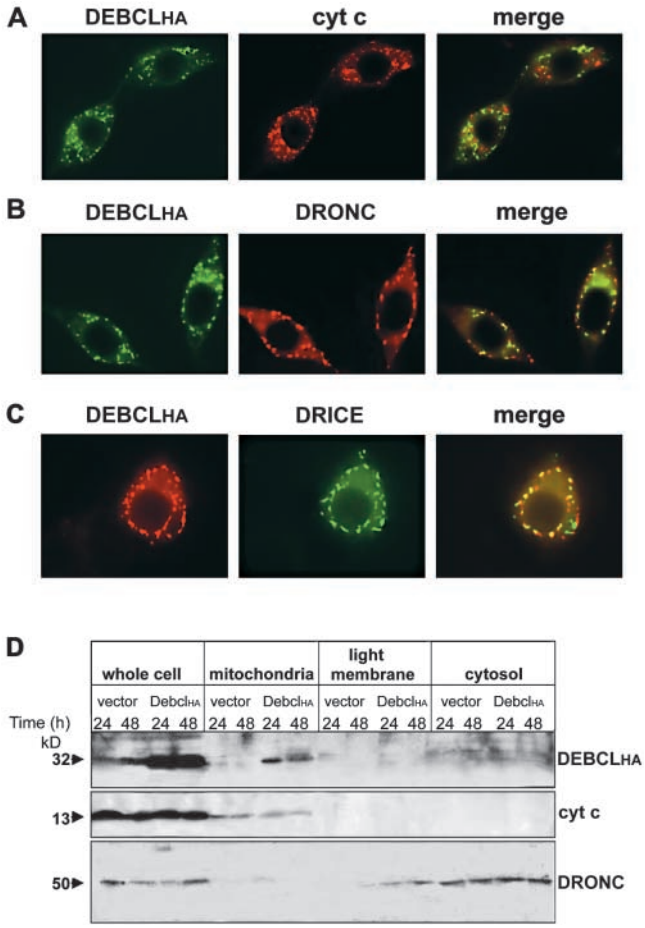

Figure 8. **Ectopic expression of DEBCL does not induce cytochrome *c* release.** BG2 cells were transfected with a DEBCL–HA expression construct. 24 h after transfection, cells were immunostained with an anti-HA monoclonal antibody and with either cytochrome *c* monoclonal antibody (A), the DRONC polyclonal 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).

cells, a large proportion of DRONC and active DRICE also colocalized with DEBCL (Fig. 8, B and C). Cell fractionation experiments confirmed that in DEBCL-transfected cells, all cytochrome *c* remains largely associated with the heavy membrane fraction (Fig. 8 D). It should, however, be noted that only ~10% of the cells are transfected with DEBCL, and at 24 h, <50% of the transfected cells were morphologically apoptotic (consistent with previously reported results with SL2 cells; Colussi et al., 2000). This may make it difficult to see small amounts of cytosolic cytochrome *c* and processed DRONC. However, active DRICE immunostaining data suggested that all DEBCL-expressing cells contain active caspase (Fig. 8 C; unpublished data).

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

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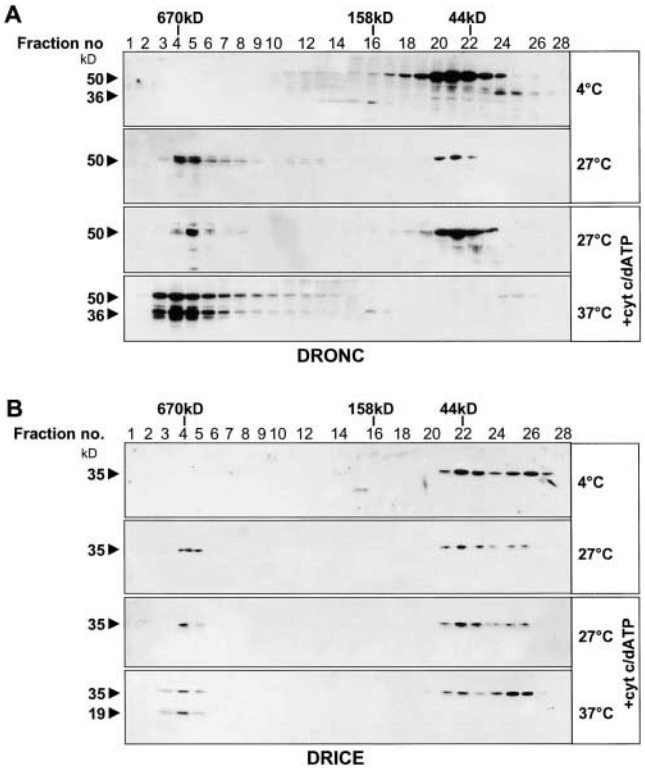

Figure 9. **Formation of large molecular complexes containing DRONC and DRICE.** BG2 cell extracts were supplemented with 2 μg cytochrome *c*, 2 mM dATP, and 1 mM $MgCl_2$ and incubated for 1 h at either 4°C, 27°C, or 37°C. Extracts were then subjected to gel filtration chromatography using a Superdex 200 column. Individual fractions were collected and subjected to electrophoresis through 15% polyacrylamide gels. Fractions were immunoblotted with anti-DRONC polyclonal antibody (A) and anti-DRICE polyclonal antibody (B), and proteins were detected by ECL. Relative positions of molecular mass markers are shown. Note that the incubation of cell extracts at 27°C or 37°C causes monomeric DRONC (50 kD) to form complexes of >700 kD. Also, recruitment of DRONC in the large complex results in its processing, as demonstrated by the presence of the 36-kD band. A fraction of DRICE, most of which is processed in extracts incubated at 37°C, is also present in the complex.

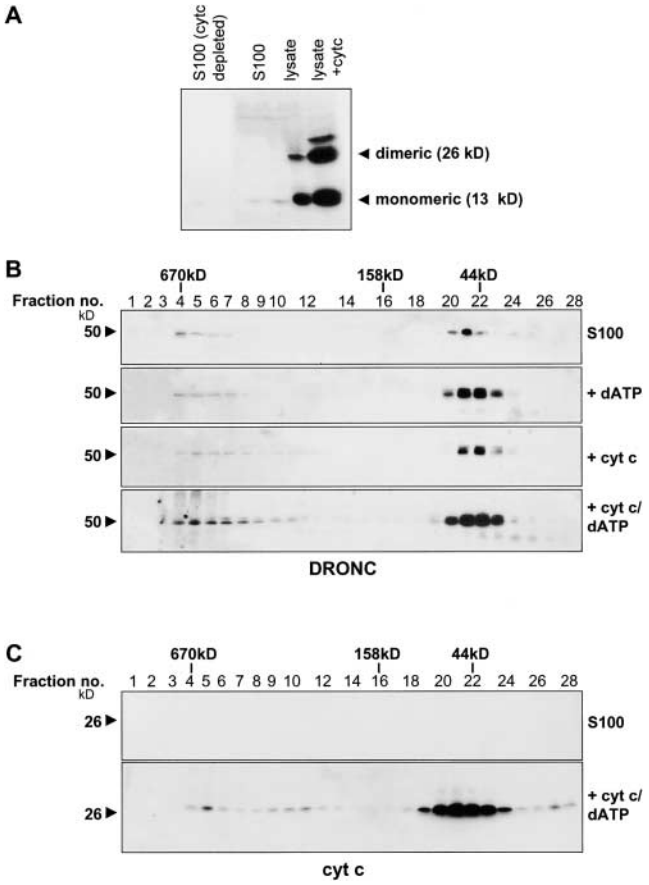

Figure 10. **Cytochrome *c* enhances formation of the >670-kD complex.** BG2 S100 fraction were immunodepleted with cytochrome *c*, as shown in A, and then incubated for 1 h at 27°C alone or supplemented with dATP, cytochrome *c*, or both dATP and cytochrome *c* (B and C). Cellular proteins were then subjected to gel filtration chromatography using a Superdex 200 column. Individual fractions were collected and subjected to electrophoresis through 15% polyacrylamide gels. Fractions were immunoblotted with anti-DRONC antibody (B) and anti–cytochrome *c* antibody, (C) and proteins were detected by ECL. Note that the immunodepleted S100 fraction lacks any detectable cytochrome *c*. Results in C show that only the dimeric (26 kD) form of cytochrome *c* is recruited to the >700-kD complex. Interestingly, a significant proportion of dimeric cytochrome *c* was detected in fractions 20–23, which corresponds to a molecular mass of ~50 kD. This species may represent the tetrameric form of cytochrome *c*, which is composed of two dimers. The monomeric form (13 kD) of cytochrome *c*, eluted in fractions 33–39 (unpublished data), was not detected in high molecular mass fractions.

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

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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, ecdysone, 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* monoclonal 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 10^6 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 ecdysone (10 μM; Sigma) for the desired time.

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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 immunoblotting 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% PEG_{4000}, 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-methylcoumaride (DEVD-amc; Enzyme System Products) or VDVAD-amc (California Peptide Research) in a total volume of 40 μl in 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 acryl cuvettes (Sarstedt), and 3 ml water was added before analysis on a luminescence 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 (37 nM) 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, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT in 250 mM sucrose and supplemented with protease inhibitor cocktail) and incubated on ice for 5 min. Cells were homogenized by 10 strokes with a tight fitting pestle, and homogenates were centrifuged twice at 500 *g* for 10 min at 4°C. Pellets, consisting of unbroken cells and nuclei, were retained 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-*S*-transferase (GST) coupled to cyanogen bromide–activated Sepharose 4 (Amersham Pharmacia Biotech) to remove GST-specific antibodies. Flowthrough from this step was affinity purified against DRONC p14–GST (Quinn et al., 2000) coupled to cyanogen bromide–activated 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 (QRSQTETD) 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 this serum with 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 7H8.2C12 (BD PharMingen), 1.5 μg/ml affinity-purified anti-DRONC rabbit antibody, 0.1 μg/ml anti-HA 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 (Amersham Pharmacia 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% propylgalate) 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 costained 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-HA high affinity rat 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.

Gel filtration chromatography

10^8 BG2 cells were resuspended in 200 μl buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 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 (Amersham 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 PharMingen). 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.

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