The two cytochrome c species, DC3 and DC4, are not required for caspase activation and apoptosis in Drosophila cells

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Introduction

Apoptosis is a highly conserved process of cell suicide that is mediated by a family of proteases known as caspases. In mammalian cells, activation of caspases can occur through two pathways (for review see Degterev et al., 2003). The death receptor pathway involves ligation of TNF family of death receptors, which results in recruitment of caspase-8 to a death-inducing signaling complex, which in turn, induces activation of caspase-8. Active caspase-8 then activates caspase-3 and other caspases by proteolytic processing. The intrinsic death pathway is induced by stimuli such as stress, UV, or cytotoxic drugs and leads to permeabilization of the mitochondrial membrane, resulting in the release of various mitochondrial proteins, such as cytochrome c, into the cytosol.

In mammals, cytochrome c triggers the activation of the apoptotic protease-activating factor (Apaf-1), enabling Apaf-1 to recruit and activate caspase-9 through a mechanism that is not completely understood (Li et al., 1997; Rodriguez and Lazebnik, 1999; Zou et al., 1999). The complex of Apaf-1, cytochrome c and active caspase-9, which is referred to as the apoptosome, induces the proteolytically processing and activation of caspase-3, the protease responsible for the majority of morphological changes associated with apoptosis (Degterev et al., 2003).

Similar to mammals, the Drosophila Apaf-1 homologue DARK, is required for activation of the Drosophila caspase-9 homologue DRONC, and apoptosis (for reviews see Kumar and Doumanis, 2000; Kumar, 2004). Like Apaf-1, DARK contains a series of WD repeats, which are the domains in Apaf-1 that interact with cytochrome c (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). One might expect that DARK, like Apaf-1, would require cytochrome c for its activation. However, whether this is true is unclear. Addition of mammalian cytochrome c to Drosophila embryonic extracts, only increased total caspase activity twofold (Kanuka et al., 1999) or had no effect on caspase activation (Varkey et al., 1999). Also, cytochrome c is not released into cytosol of Drosophila cells during apoptosis, even though DRONC is activated (Varkey et al., 1999; Dorstyn et al., 2002). These findings led to the proposal that cytochrome c undergoes a conformational change on the surface of the mitochondrial membrane, and that this change is required and sufficient for the activation of DARK (Varkey et al., 1999). However, silencing the expression of the cytochrome c proteins had no effect on apoptosis of Drosophila SL2 cells (Zimmermann et al., 2002). Given that apoptosis can proceed in the absence of cytochrome c proteins, these results suggest that cytochrome c is not important for cell death or caspase activation in Drosophila cells.
Figure 1. DC3 and DC4 are not required for caspase activation and apoptosis. (A) Endogenous expression of dc3 and dc4 transcripts in Drosophila cell lines as detected by RT-PCR. (B) BG2 cells were treated with dsRNAs for dc3, dc4, or both dc3 and dc4, and abolition of the target genes assessed by RT-PCR. Amplification of a 403-bp dronc region was used as a control. (C) BG2 cells treated with dsRNA to dc3, dc4 or both, were exposed to cycloheximide (CHX) or were left untreated. Absence of DC4 protein was confirmed by immunoblotting with cytochrome c antibody. Processing of DRONC and DRICE was detected by immunoblotting. Relative molecular masses of the proteins in kilodaltons are shown. (D) Apoptosis induced in BG2 cells by CHX (10 μg/ml for 6 h), or l(2)mbn cells by ecdysone (10 μM for 24 h) treatment was estimated by scoring cells with condensed nuclei by DAPI staining. Data (mean ± SEM) were derived from four independent experiments. (E) Caspase activity, presented as relative fluorescence units (RFU), was determined on DEVD-amc substrate in BG2 or l(2)mbn cells treated (+) with CHX [10 μg/ml for 6 h] or ecdysone [10 μM for 24 h], respectively, or left untreated (−). Data (mean ± SEM) were derived from three experiments.

Drosophila encodes two cytochrome c proteins, DC3 and DC4 (Limbach and Wu, 1985). DC4 has highest homology to cytochrome c from other species, and its expression is abundant but varies in different tissues with stage of development. DC3 is ubiquitously expressed during development but at much lower levels (Limbach and Wu, 1985). DC3 has been implicated in the activation of caspases during spermatid individualization in Drosophila (Arama et al., 2003). These observations raise the possibility that DC3 can function as an activator of DARK at some stages of Drosophila development. Here, we have investigated whether either DC3 or DC4 proteins are required for activation of caspases in Drosophila, and if they can induce caspase activation in a cell-free system. Our data indicate that both cytochrome c species are not required for caspase activation in Drosophila development and in Drosophila cells, even though they can induce activation of Apaf-1.

Results and discussion

DC3 and DC4 are not required for caspase activation in Drosophila cells

To compare the expression of dc3 and dc4 in Drosophila cell lines, we used RT-PCR analysis and found that dc3 is expressed at lower levels in BG2, SL2 and l(2)mbn cells, compared with dc4 (Fig. 1 A). Expression of dc3 was the lowest in BG2 cells. To test whether DC3 or DC4 are required for the activation of DRONC or DRICE, we silenced the expression of dc3 and dc4 by RNA interference (RNAi) in Drosophila BG2 cells, with double-stranded RNAs (dsRNAs) to each gene. The ablation was confirmed by RT-PCR for both dc3 and dc4 (Fig. 1 B) and by immunoblotting for DC4 (Fig. 1 C). Silencing both dc3 and dc4 in BG2 cell had no effect on the processing of DRONC and DRICE after induction of apoptosis by cycloheximide (Fig. 1 C), the rate of cell death (Fig. 1 D), or caspase activity (Fig. 1 E). We also found that silencing dc3 and dc4 did not affect ecdysone-induced apoptosis (Fig. 1 D) or caspase activity (Fig. 1 E) in Drosophila l(2)mbn cells. These observations are consistent with previous studies (Zimmermann et al., 2002), which demonstrated that silencing expression of cytochrome c proteins in SL2 cells had no effect on apoptosis induced by UV, actinomycin D, or ectopic expression of Reaper or Grim. These results clearly indicate that DC3 and DC4, are not essential for caspase activation and apoptosis in Drosophila cells.

DRONC processing is not enhanced by overexpression of DC3 or DC4

Although Drosophila cytochrome c proteins are not required for caspase activation and apoptosis, it is possible that they can induce caspase activation but can be substituted by some redundant activity. We tested this possibility in cultured cells and in cell-free systems. One approach was to test whether overexpression of the cytochromes in BG2 cells had any effect on cell death. We generated BG2 cell lines that expressed DC3 or DC4 protein fused with the HA epitope tag and confirmed expression of proteins by immunoblotting (Fig. 2 A). Ectopic expression of DC3 or DC4 did not induce processing of DRONC or DRICE. When these cells were treated with cycloheximide, we found that cells expressing exogenous DC3 or DC4 underwent apoptosis at the same rate as the parental cells (Fig. 2 B) and showed caspase activity comparable to controls (Fig. 2 C).

We had demonstrated previously that cytochrome c is not released from mitochondria in Drosophila cells (Dorstyn et al., 2002). The failure to observe cytochrome c release could be interpreted as a failure of cytochrome c to leave mitochondria or a failure to detect the released protein. We decided to take advantage of the cell lines that ectopically expressed DC3 or DC4 to test whether these proteins, particularly DC3 which is not normally abundant in BG2 cells, are released during apoptosis. We treated cells with cycloheximide and fractionated them by differential centrifugation at various times after treatment. We found
the majority of both DC3 and DC4 were in the heavy membrane fraction that contained mitochondria (Fig. 2 D). Interestingly, DC3, but not DC4 was also detectable in the cytosolic fraction of both normal and apoptotic cells. Both DC3 and DC4 were detectable in the light membrane fractions comprising Golgi and ER, but there was no significant change in protein levels after treatment (unpublished data). It is unclear why DC3 is present in cytosol in healthy cells. This may simply be an artifact of overexpression, or likely represents a portion of apocytochrome c that is unable to enter the mitochondria. However, it is clear that the presence of DC3 in cytosol does not affect DRONC activation or apoptosis. DRICE activation was also unaffected in these cells (unpublished data). We conclude that although DC3 and DC4 are not released into the cytoplasm during apoptosis, DC3...
possibly resides in both cytoplasm and mitochondria of *Drosophila* cells but cannot induce caspase activation and apoptosis.

To verify the results obtained by cell fractionation, we assessed the localization of DC3 and DC4 in these BG2 cell lines. We found that the majority of DC3 and DC4 protein colocalize with MitoTracker, in both live and apoptotic cells (Fig. 3 A), indicating that these proteins remain in the mitochondria during apoptosis. A portion of DC3 was detectable outside of mitochondria in live and apoptotic cells (Fig. 3 A) consistent with its detection in light membrane and cytosol fractions (Fig. 2 D). We used an antibody that detects processed DRICE protein and confirmed that ectopic expression of DC3 or DC4 did not cause DRICE activation. Consistent with our previous observations (Dorstyn et al., 2002) we also found that in cells treated with cycloheximide, active DRICE partially colocalized with mitochondria (Fig. 3 C). However, considering that neither of the cytochromes are required for DRICE activation, the observed association may be unrelated to caspase activation.

**Recombinant DC3 and DC4 do not promote caspase activation in cell extracts**

We set out to test whether DC3 or DC4 can induce caspase activation in a cell-free system. Recombinant DC3 and DC4 were generated by coexpressing these proteins with haem lyase in *E. coli*. The absorbance spectrum of the purified recombinant DC3 and DC4 proteins showed peaks at 419, 520, and 549 nm, similar to the spectrum shared by human cytochrome *c* (Fig. 4 A). The identity of these purified proteins was confirmed by immunoblotting with cytochrome *c* antibody (Fig. 4 B). We added recombinant DC3 or DC4 to S100 extracts from *Drosophila* BG2 or mammalian 293T cells that do not contain endogenous cytochrome *c* (Fig. 4 C). We found that the addition of DC3 and DC4, in the presence of dATP, did not significantly increase caspase activity in *Drosophila* BG2 cells (Fig. 4 D). Interestingly, if we used apoptotic extracts from BG2 cells we detected a small but significant increase in caspase activity after the addition of either *Drosophila* or human cytochrome *c* (unpublished data). When apoptotic extracts from cells subjected to *dc3* and *dc4* RNAi were used, the increase in caspase activity was still evident upon addition of recombinant cytochrome *c* proteins (unpublished data). These results indicate that additional apoptotic factors may augment caspase activation in the presence of cytochrome *c*. In contrast, we could clearly see that both DC3 and DC4 induced caspase activity in mammalian 293T extracts (Fig. 4 D). This activity is comparable to the increase in caspase activity seen when purified human cytochrome *c* is added. Interestingly, human cytochrome *c* was unable to induce caspase activity in BG2 cell extracts. These data indicate that whereas cytochrome *c* is necessary for caspase activation in mammalian cells, it does not directly activate caspases in *Drosophila* cells.

**DC3 and DC4 are not essential for caspase activation in vivo**

To examine whether DC3 or DC4 is required for cell death in *Drosophila* development, we assessed whether lysates from *dc3* and *dc4* loss of function mutants were deficient in caspase activation. Two independent P-element mutations in each of the cytochrome *c* genes and a deficiency in this region have been reported previously (Arama et al., 2003). A P-element insertion in *dc4* ([l(2)K13095 allele]) is homozygous lethal, which is likely due to its important role in respiration. The P-element insertion, which disrupts *dc3* (*bln*1 allele) is homozygous viable but results in male sterility (Castrillon et al., 1993; Arama et al., 2003). Recent studies have shown that the *bln*1 insertion results in significantly reduced levels of activated DRICE in spermatids (Arama et al., 2003). Both P-element insertions, however, disrupt multiple genes of unknown function (Huh et al., 2004). Therefore, it remains unclear whether DC3 is required for DRICE activation. A deficiency in the *dc3* and *dc4* gene region (Df(2L)H20) has a homozygous lethal phenotype as expected, but also disrupts the expression of multiple genes located within this region, making it difficult to attribute a function to either *dc3* or *dc4*.

Pre-pupae were collected 4 h after puparium formation, a time when various larval tissues including salivary glands undergo cell death and *dronc* has been shown to be up-regulated (Cakouros et al., 2002, 2004). We prepared lysates from *bln*1...
heterozygote (dc3+/−) and homozygous (dc3−/−) mutant prepupae, l(2) k13095 heterozygotes (dc4+/−) and from prepupae heterozygous for the deficiency, Df(2)H20 (Df(2)Δdc3/dc4). As shown in Fig. 5 A, there was a high level of caspase activity in wild-type (W1118) prepupae. There was no reduction in the level of caspase activity in pupae from dc3 and dc4 mutants (Fig. 5 A) or in the processing of DRONC (Fig. 5 B). Animals heterozygous for the deficiency Df(2)H20 also did not show lower caspase activity, even though the expression of cytochrome c in these flies is clearly reduced (Fig. 5 B). In addition, TUNEL staining of homozygous mutant embryos did not show any decrease in developmental cell death (unpublished data).

Conclusions and perspective

Our data clearly show that neither of the two cytochrome c species in Drosophila are required for caspase activation or apoptosis. Previous studies reported that a P-element insertion in the dc3 gene (bln1) results in loss of DRICE activity in testis (Arama et al., 2003). However, a recent report indicates that the bln1 P-element insertion also disrupts a number of other genes (Huh et al., 2004), thus questioning whether DC3 is responsible for DRICE activity. Additionally, DRICE activation during spermatogenesis appears to be independent of DARK and DRONC (Huh et al., 2004). If DC3 is required for caspase activation in Drosophila, a loss of function mutation in dc3 should lead to severe developmental defects and lethality. Furthermore, although a tissue-specific function has been suggested for DC3, it is unlikely that DC3 functions only during spermatogenesis given its ubiquitous expression. Although disruption of the dc4 gene is embryonic lethal, we have shown that DC4 cannot induce caspase activation and apoptosis in Drosophila cells.

The question remains as to how DARK mediates DRONC activation. One possibility is that other factors can substitute for cytochrome c function during apoptosis. Alternatively, removal of DIAP1 from DRONC may be sufficient to allow an interaction with DARK and activation. Given that transcription plays a major role in developmental PCD in Drosophila, changes in the concentration of DIAP1, DRONC, and DARK proteins could facilitate caspase activation in the fly (Kumar and Cakouros, 2004). Our studies, combined with published work, demonstrate that Drosophila and mammalian cytochrome c proteins are functionally similar as they can both mediate respiration and Apaf-1 activation in mammalian cell lysates. Therefore, the requirement for cytochrome c in caspase activation in mammals is likely to have evolved late in evolution.

Materials and methods

Constructs

Full-length dc3 and dc4 cDNAs were PCR amplified with a 3′ HA-tag, from a Drosophila larval cDNA library, and cloned into pIE1.4 vector (Novagen). For bacterial expression, dc3 and dc4 cDNAs were cloned into pQCI.2 backbone (provided by A. Rodriguez, The Rockefeller University, New York, NY, H. Mizuno, Massachusetts Institute of Technology, Cambridge, MA, and A. Matapurkar, Cold Spring Harbor Laboratory) along with cDNA encoding hem globin.

Cell culture

Drosophila Ml-DmbB2c2 (BG2) or I2mbm cells were maintained at 27°C as described previously (Cakouros et al., 2002; Dorstyn et al., 2002). DC3 and DC4 expressing cell lines were generated by transfecting BG2 cells with pIE1.4-DC3HA, or pIE1.4-DC4HA together with a pH5-Neo expression construct (provided by N. Silverman, University of Massachusetts Medical School, Worcester, MA) as described previously (Cakouros et al., 2004). Transfected cells were selected with 1 mg/ml G418 (GIBCO BRL). Cells were induced to undergo apoptosis as described previously (Cakouros et al., 2002; Dorstyn et al., 2002). Cell viability/death was assessed by Trypan blue exclusion and staining with DAPI.

Fly strains

Drosophila wild-type (W1118), dc3 “blanks” mutants ([bln+/bln+], [bln+/CyoKr->GFP], dc4 mutants ([l2(1) k13095/CyoKr->GFP], and dc3/dc4 deficiency strains (Df(2R) H20/CyoKr->GFP) were obtained from Bloomington Stocks Center.

Cytochrome c expression and purification

E. coli DH5a cells were transformed with either pQCI.2-DC3, pQCI.2-DC4, or pQCI.2-hcyc expression constructs. 500-ml cultures were induced with 1 mM IPTG overnight at 37°C. Cells were lysed by sonication and cleared lysates were incubated with 300 μl prewashed Ni-NTA agarose beads [Qiagen] for 1 h at 4°C. Beads were washed with 50 vol lysis buffer and cytochrome c proteins eluted with 5 vol elution buffer [300 mM NaCl, 10 mM Tris-HCl, pH 8, 500 mM imidazole].

Caspase assays

Fluorogenic substrate assays were performed as described previously (Dorstyn et al., 1999). Cytosolic S100 protein lysates were isolated as reported previously (Dorstyn et al., 2002). Pre-pupa were snap frozen in liquid nitrogen and lysed by homogenisation. 20–50 μg of lysates or S100 were used in DEVD- or VDVAD-amc cleavage reactions. Where indicated, S100 lysates were incubated with 10 μM of purified DC3mut, DC4mut, or human cytochrome c (hcyc), and 1 mM dATP, in caspase assay buffer (Dorstyn et al., 1999) at RT and activity was measured over time using a fluorometric plate reader (PerkinElmer) (excitation 385 nm, emission 460 nm).

Cell fractionation

Mitochondria and cytosol from untreated and cycloheximide (15 μg/ml)-treated cells were separated by differential centrifugation as described previously (Dorstyn et al., 2002).

Immunoblotting

Aliquots (50 μg protein) were immunoblotted using the following antibodies: monoclonal cytochrome c 7H8.2C12 (PharMingen) used at 0.5 μg/ml, rat HA (Roche) used at 0.1 μg/ml, rabbit DRONC 1.5 μg/ml, and rabbit DRICE (provided by B. Hay, California Institute of Technology, CA) at 1 μg/ml (Dorstyn et al., 2002; Huh et al., 2004).
Immunofluorescence
Cells were stained as described previously (Dorstyn et al., 2002). Where indicated, cells were incubated with 1 μg/ml HA antibody alone or together with DRICE antibody at 4 μg/ml overnight at 4°C followed by incubation with Alexa Fluor 488 (green), Alexa Fluor 568 (red) conjugated anti–rabbit or goat anti–rat Texas red secondary antibodies (Molecular Probes). Fluorescence images were captured using a fluorescence microscope (model BX51; Olympus) and a camera (model U-CCD3/CV-M300; Olympus). Cells were visualized under 100× UPLAPO objective lens with NA = 1.3, under oil immersion. All green and red fluorescent images were automatically merged. Image analysis was performed with MetaSystems his software (Robert Bosch). Image background brightness was adjusted to vector control cells. Images were compiled using Adobe Photoshop version 6.0.

RNAi
dc3 and dc4 were cloned into pCDNA3 (Invitrogen) and a 700-bp coding region of GFP was cloned into pGEM-T Easy vector (Promega) for use as a control. dsRNA was generated as described previously (Colussi et al., 2000; Cakouros et al., 2002). 40 nM of dsRNA was added to 2 × 10^6 cells in 1 ml serum-free medium. In the case of coablation of dc3 and dc4, 40 nM of each dsRNA reactions were mixed and added to cells. Cells were then incubated for 1 h at 27°C after which 2 ml of complete media containing 10% FBS was added. Cells were incubated over 48 h and treated with cycloheximide [10 μg/ml] for 6 h, or with edcsyne (10 μM) for 24 h.

RT-PCR
Total RNA was used for RT-PCR. To assess ablation of dc3 and dc4, transcripts were amplified using a 5′ primer designed to 55 bases upstream of the ATG, and a 3′ primer designed to the terminus of each transcript. Amplification of dronc was performed using primers designed from the 5′ ATG and a 3′ primer designed to a region 400 bp downstream of the start ATG.

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