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Mitochondrial regulation of insect cell apoptosis: Evidence for permeability transition pore-independent cytochrome-*c* release in the Lepidopteran Sf9 cells

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ABSTRACT

Role of cytochrome-*c* in insect cell apoptosis is highly controversial, with many earlier reports suggesting lack of involvement of mitochondrial factors in *Drosophila* while more recent studies have indicated otherwise, thus warranting more in-depth studies of insect cell apoptosis. In the present study, we investigated mitochondrial involvement during actinomycin-D induced apoptosis in Sf9 Lepidopteran cells. Cytochrome-*c* was released from mitochondria very early during apoptosis, and was preceded quickly by ROS generation and cardiolipin peroxidation. Albeit cytochrome-*c* release and apoptosis induction were inhibited by bongkrkicacid (BKA) it appears that the release is independent of permeability transition pore (PTP) as it preceded mitochondrial Ca^{2+} buildup and mitochondrial membrane potential (MMP) loss. Further, the release was found to be unaffected by PTP inhibitor cyclosporin-A. Bax inhibitory peptide BiP-P5 could effectively block both cytochrome-*c* release and apoptosis induction indicating dependence on Bax-channel formation. Inhibition of apoptosis by FSBA, a nucleotide analog that inhibits apoptosome formation through Apaf1 binding, suggested activity of apoptosome similar to mammalian cells. Mitochondria isolated from treated cells activated caspases in the cytosolic fraction of untreated cells while mitochondrial lysates of treated or untreated cells had similar effect. Sequestering cytochrome-*c* in mitochondrial lysates inhibited DEVDase activity, and addition of purified cytochrome-*c* and dATP to Sf9 cytosolic fraction induced DEVDase activity, suggesting that cytochrome-*c* may be exclusively required for Lepidopteran apoptosis. This is the first detailed study demonstrating mitochondrial regulation of Lepidopteran insect cell apoptosis, and reiterates its homology with mammalian cell apoptosis while showing distinctive differences from earlier reports in *Drosophila*.

1. Introduction

Apoptosis is an evolutionarily conserved form of programmed cell death to remove surplus or unwanted cells in metazoans (Ellis et al., 1991). In the intrinsic pathway of apoptosis, mitochondria sense catastrophic cellular changes and irreversibly commit cells to apoptosis by releasing death factors such as cytochrome-*c*, a soluble protein, into cytosol (Kroemer et al., 1995). Once released into the cytosol, cytochrome-*c* binds with Apaf-1, inducing a conformational change in the latter that facilitates binding of this complex with pro-caspase-9 and dATP, resulting in non-proteolytic cleavage of caspase-9 (Stennicke et al., 1999), and subsequently leads to a cascade of caspase activation. Although the mechanisms of cytochrome-*c* release are not yet fully understood, two distinct phenomena leading to cytochrome-*c* release have been thoroughly investigated. While one of the mechanisms is dependent on formation of mitochondrial permeability transition pore (PTP) and is mediated by Ca^{2+} overload, the second mechanism is PTP-independent and is mediated by Bcl2 family proteins. A third mechanism of cytochrome-*c* release which is independent of both PTP and Bcl2 family proteins but mediated by serine proteases has also been recently suggested, thus displaying the complex nature of this process (Mizuta et al., 2007). The role of cytochrome-*c* in intrinsic pathway of mammalian cell apoptosis has been well established by various studies, including the ones that showed that addition of cytochrome-*c* and dATP is sufficient for caspase-3 activation in cell-free systems (Liu et al., 1996) and deficiency of cytochrome-*c* attenuated the stress-induced apoptosis (Li et al., 2000).

A majority of studies on apoptotic regulation in insects have been carried out using the *Drosophila* system. Although apoptotic events are evolutionarily conserved, some fundamental differences between the Drosophila and mammalian apoptotic signaling have been reported. In mammals, caspase activation is the ultimate step after which cell death becomes irreversible, whereas in Drosophila,

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apical caspase DRONC (a homologue of mammalian caspase-9) is chronically activated in most of the cells. In normal conditions the DRONC activity is suppressed by DIAP1, thereby allowing cells to survive. During caspase-dependent cell death, disruption of DIAP-1-DRONC interaction is achieved by several pro-apoptotic proteins such as RPR, HID, GRIM, SKL and JAFRAC (Yoo et al., 2000) setting activated DRONC free. Activation of DRONC is mediated by DARK/Dapaf-1/HAC-1, which is a homolog of CED-4/Apaf-1. Although Dark is necessary for cell death, cytochrome-*c* protein has been reported to be dispensable for caspase activation in the apoptotic program of *Drosophila* (Dorstyn et al., 2002, 2004; Dorstyn and Kumar, 2006; Varkey et al., 1999). Neither of the two *Drosophila* cytochrome-*c* species, DC3 and DC4 (proximal and distal, respectively) were observed to release from mitochondria into cytosol, and addition of mammalian cytochrome-*c* to *Drosophila* embryo extracts did not affect caspase activation (Varkey et al., 1999). Similarly, silencing of cytochrome-*c* expression did not affect the induction of apoptosis in *Drosophila* S2 cells (Zimmermann et al., 2002), and absence of involvement of mitochondrial factors was further confirmed using the *Drosophila* cytosolic extracts (Means et al., 2006).

While a number of these studies have shown that mitochondrial factors may not be involved in *Drosophila* apoptosis, the existence of Bcl2 family proteins in Drosophila including the Bax homologue that translocates to mitochondrial membrane (Zhang et al., 2000) indicates that further investigations may be required to unequivocally establish this fact. Indeed, certain recent reports have provided contrasting evidence suggesting a possible role of mitochondrial factors during apoptosis in Drosophila (Abdelwahid et al., 2007; Goyal et al., 2007). Therefore, mitochondrial mechanisms in insect cell apoptosis warrant further detailed investigations. Using additional model systems besides *Drosophila* would help understand the intricacies of cell death mechanisms in insect cells. Lepidoptera (butterflies and moths), a prominent order within the class Insecta, is an important model system for studying cell death mechanisms due to being evolutionarily close to *Drosophila* (order Diptera), as well as due to its exemplary resistance against many stress agents including ionizing radiation and alkylating mutagens (Chandna et al., 2004; Koval, 1994). Investigations on Lepidopteran cell death mechanisms have revealed the presence of certain caspases that show good structural and functional homology with mammalian system. Clem and Miller (1994) and Seshagiri and Miller (1997) have shown that actinomycin-D induces extensive apoptosis in Lepidopteran cells, which is mediated by caspase-3. In the present study, we investigated mitochondrial mechanisms involved in actinomycin-D induced apoptosis in Sf9 cell line, including the regulation of cytochrome-*c* release and the dependence of downstream activation of caspases on this event. While our study reinforces an important role for cytochrome-*c* in the Lepidopteran apoptosis, it also provides important evidence for the existence of PTP-independent cytochrome-*c* release in Sf9 cells. Further, this study shows that caspase activation in Sf9 cell extracts is exclusively dependent on the release of cytochrome-*c*.

2. Materials and methods

2.1. Fluorescent probes and chemicals

All fluorescent probes including calcein-AM, 5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), nonyl acridine orange (NAO), propidium iodide, and rhodamine-123 were obtained from Molecular Probes and anti-cytochrome-*c* antibodies with broad species specificity, fluorigenic caspases-3 substrate (Ac-DEVD-AMC) and caspases-3 inhibitor (Ac-DEVD-FMK) were obtained from BD-pharmingen. Calcium ionophore A23187, purified cytochrome-*c*, 5′-(4-fluorosulfonylbenzoyl) adenosine hydrochloride (FSBA), bongkrekic acid (BKA), cyclosporin A (Cs-A) and actinomycin-D were purchased from Sigma-Aldrich. Bax inhibiting peptide, Pro-Met-Leu-Lys-Glu, (BIP) P5 and negative control peptide, Ile-Pro-Met-Ile-Lys, were obtained from Tocris.

2.2. Cell culture

Sf9 cells (established from ovaries of *Spodoptera frugiperda*; gifted by Dr. SE Hasnain, Hyderabad Central University, Hyderabad, India) were maintained as monolayers in 25-cm^2 culture flasks at 28 °C in Grace's insect cell medium (GibcoBRL) supplemented with 3.33 g l^{-1} lactalbumin hydrolysate, 3.33 g l^{-1} yeastolate, 0.35 g l^{-1} $NaHCO_3$ and antibiotics (Penicillin-sodium salt 50,000 units/l, streptomycin sulfate 50,000 μg/l, Nystatin 2000 μg/l from 500,000 USP Units/mg; Sigma, USA). Growth medium (pH 6.2) was prepared by adding 10% heat inactivated FCS (Sigma, USA) and stored below 8 °C. Cells were regularly subcultured twice a week in exponential phase and by seeding at a density of 35,000–40,000 cells/cm^2 as described earlier (Chandna et al., 2004). Cells were treated with 0.5 μg/ml actinomycin-D (1 mg/ml stock solution prepared in water) as indicated in Section 3.

2.3. Live cell morphology and apoptosis

Apoptotic body formation was studied by Nomarski-DIC time-lapse microscopy using the Axiovert 200 Zeiss inverted microscope (Carl Zeiss, Germany) and apoptotic DNA degradation was studied by DNA and single cell gel electrophoresis or comet assay. For DNA gel electrophoresis, cells were lysed for 1 h at 50 °C with lysis buffer containing 10 mM EDTA, 0.5% SDS, 50 mM Tris, 0.5 μg/ml proteinase K. Following heat inactivation of proteinase K at 75 °C for 15 min, RNA was degraded with RNase A for 1 h at 37 °C. Whole cell lysate was electrophoresed on 1.2% agarose gel (~8.00 mm thick) containing ethidium bromide. Single cell gel electrophoresis was performed as described earlier (Chandna, 2004). After completing the treatments, approximately 30,000 cells were mixed with pre-warmed 0.75% ultra low gelling agarose and layered on microscopic slides which are pre-coated with 0.1% agarose. After allowing the formation of agarose gel at 4 °C, cells were lysed by submerging the slides in lysis buffer containing 2.5% sodium dodecylsulfate, 1% sodium sarcosinate, and 25 mM EDTA, pH 9.5 for 15 min at room temperature. Slides were then washed for 5 min in distilled water at 10 °C and electrophoresed in electrophoresis buffer containing 90 mM Trizma base, 90 mM boric acid and 2.5 mM EDTA, pH 8.3 at 2 V/cm for 5 min at 10 °C. After a brief rinse in distilled water, slides were air dried at 45 °C on a hot plate and stored in a cool and humid environment until use. Comets were stained with 50 μg/ml propidium iodide after rehydration of slides and observed under fluorescence microscope. Apoptotic comets were scored as described earlier (Chandna, 2004).

2.4. Measurement of intracellular ROS

Intracellular ROS levels were measured using 5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Lebei et al., 1992). After the treatment, cells were washed twice with PBS and held at 37 °C in PBS containing Ca^{2+}, Mg^{2+} and H_2DCFDA (10 μg/ml; Merck, England). After 30 min, cells were washed again and analyzed by flow cytometry.

2.5. Measurement of mitochondrial membrane potential (MMP) and cardiolipin peroxidation

Changes in mitochondrial membrane potential and cardiolipin peroxidation were studied by Rhodamine-123 and N-nonyl-acridine orange (NAO) staining respectively as described by