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Research paper

Cloning and functional characterizations of an apoptogenic *Hid* gene in the Scuttle Fly, *Megaselia scalaris* (Diptera; Phoridae)

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ABSTRACT

Although the mechanisms of apoptotic cell death have been well studied in the fruit fly, *Drosophila melanogaster*, it is unclear whether such mechanisms are conserved in other distantly related species. Using degenerate primers and PCR, we cloned a proapoptotic gene homologous to *Head involution defective* (*Hid*) from the Scuttle fly, *Megaselia scalaris* (*MsHid*). *MsHid* cDNA encodes a 197-amino acid-long polypeptide, which so far is the smallest HID protein. PCR analyses revealed that the *MsHid* gene consists of four exons and three introns. Ectopic expression of *MsHid* in various peptidergic neurons and non-neuronal tissues in *Drosophila* effectively induced apoptosis of these cells. However, deletion of either conserved domain, N-terminal IBM or C-terminal MTS, abolished the apoptogenic activity of MsHID, indicating that these two domains are indispensable. Expression of *MsHid* was found in all life stages, but more prominently in embryos and pupae. *MsHid* is actively expressed in the central nervous system (CNS), indicating its important role in CNS development. Together MsHID is likely to be an important cell death inducer during embryonic and post-embryonic development in this species. In addition, we found 2-fold induction of *MsHid* expression in UV-irradiated embryos, indicating a possible role for *MsHid* in UV-induced apoptosis.

1. Introduction

Programmed cell death (PCD) in eukaryotic cells is an active mechanism that eliminates damaged or infected cells, supernumerary cells during embryonic development, and obsolete cells during post-embryonic development. In those cells that are fated to die, genetic program is turned on in response to a developmental signal, pathogenic stimuli or DNA damage (Danial and Korsmeyer, 2004).

Apoptosis is a type of PCD that is characterized by chromatic condensation, formation of membrane blebs, and DNA breaks (Elmore, 2007). Such cytological symptoms are mainly catalyzed by a group of proteolytic enzymes, namely caspases (Hay and Guo, 2006). Caspases are classified into two groups: initiator and effector. The former enzymes activate the latter ones to amplify overall activities, which degrade cellular contents rapidly, thereby inducing irreversible cell death.

Extensive genetic analyses done in the fruit fly, *Drosophila melanogaster*, identified several important molecular components that are associated with the regulation of caspase activities (Hay and Guo, 2006). In live cells, caspase activities are held in check via physical interaction with endogenous caspase inhibitors, called DIAP1 (*Drosophila* inhibitor of apoptosis 1). In response to death signals, DIAP1 is antagonized by a group of proapoptotic gene products, Reaper, HID, Grim or Sickle, which are often collectively referred to as RHG family (White et al., 1994; Kornbluth and White, 2005). These death inducers interact with DIAP1 through their conserved IAP-binding motif (IBM), causing displacement of DIAP1 from caspases and auto-degradation of it. Despite such consensus molecular event, tissue-specific apoptosis is mediated by different cell death genes. For instance, metamorphosis-associated degeneration of the salivary glands requires *reaper* and *Hid* (Jiang et al., 2000), while *Hid* is necessary for cell death in response to the depletion of the survival signal (Kuroda and White, 1998; Bergmann et al., 2002). On the other hand, we and others have shown that *grim* is the principal death inducer for developmentally-regulated PCD of larval neurons producing Corazonin, bursicon, and CCAP neuropeptides (Draizen et al., 1999; Lee et al., 2013a, 2013b).

To date, only a few non-drosophilid *RHG* homologous genes have been characterized: *michelob_x* in several mosquito species (Zhou et al., 2005) and *IAP-antagonist michelob like protein* (*IMP*) in the yellow fever mosquito *Aedes aegypti* (Bryant et al., 2008); a *reaper*-like *IAP-*

Abbreviations: Hid, Head involution defective; PCD, programmed cell death; CNS, central nervous system; DIAP, *Drosophila* inhibitor of apoptosis; IBM, IAP binding motif; MTS, mitochondrial targeting sequence; RHG, Reaper, Hid, Grim; RGS, Reaper, Grim, Sickle; APF, after puparium formation.

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binding motif 1 (*ibm1*) in the silkworm *Bombyx mori* (Bryant et al., 2009; Zhang et al., 2010; Wu et al., 2013); a *reaper* in the blow fly, *Lucilia cuprina* (Chen et al., 2004); *reaper* and *Hid* in the Caribbean fruit fly, *Anastrepha suspensa* (Schetelig et al., 2011); *reaper* and *Hid* in the European green blow fly, *Lucilia sericata* (Edman et al., 2015). Despite rapid expansion of the Arthropod genome project (i5K Consortium, 2013), difficulties in identifying *RHG* homologous genes are due to poor conservation of the amino acid sequences of the death inducers, except for a short conserved IBM. Alternatively, *RHG* genes may not be major death inducers in other insect groups. Therefore, identification and characterization of *RHG* homologs is an important step in understanding the evolution of apoptotic mechanisms.

To address this question, we employed the Scuttle fly, *Megaselia scalaris* (Order Diptera; Cyclorrhapha; Aschiza; Platypezoidea; Phoridae) as a target organism. This group, along with syrphid flies, belongs to Section Aschiza, which diverged from the *Schizophora* lineage about 150 million years ago (Wiegmann et al., 2011). The Schizophora section includes >50% of dipteran families, such as Drosophilidae, Tephritidae, Muscidae and Calliphoridae. The main difference between Aschiza and Schizophora is the absence (Aschiza) or presence (Schizophora) of the ptilinum in the head (http://www4.ncsu.edu/unity/lockers/ftp/bwiegman/fly_html/diptera.html).

M. scalaris, along with other related species, is also known as 'coffin flies' reflecting their remarkable ability to infest corpses within coffins. The larvae are omnivorous feeder on various organic matters including dead animals and other decaying matters. Therefore, they have been used in forensic science to estimate postmortem intervals particularly in tightly enclosed containments (Disney, 2008; Reibe and Madea, 2010). They are also facultative parasites causing Myiasis (infestation of living tissue with maggots) in animals including humans (Disney, 2008; Hira et al., 2004; Ghavami and Djalilvand, 2015; Vanin et al., 2013). Recent advancements in genomic studies and the convenience of maintaining colonies in the laboratory make this and related species (*M. abdita*) as an emerging model system in evolutionary biology (Rafiqi et al., 2011; Wotton et al., 2014). For instance, distinct sex-determination pathway found in *M. scalaris* provided insight into the diversified mechanisms of common biological events in insects (Kuhn et al., 2000; Sievert et al., 2000; Verhulst et al., 2010; Hoehn and Noor, 2015). Whether this is the case for the apoptotic cell death is one of our long-term research interests.

Here we report the isolation and molecular characteristics of a *Hid*-homologous gene in the Scuttle fly (*MsHid*). Despite significant structural deviation from other HID homologs, transgenic expression of *MsHid* in various cells in *D. melanogaster* induced cell death, suggesting a conserved apoptogenic function of Ms-HID.

2. Materials and methods

2.1. Scuttle fly maintenance

Scuttle flies, *M. scalaris*, were locally collected from garbage and domesticated in house-made media (rodents' pellet-agar-yeast-Tegosept) at room temperatures (20–24 °C). The food recipe is available upon request.

2.2. 3′-Rapid amplification of cDNA end (RACE)

Sequences of PCR primers are presented in Table 1. Total RNA was isolated from twelve 0 to 24-hour-old pupae. The pupae were homogenized in 500 μL of Trizol (Life Technologies) and then RNA was purified as per the manufacturer's protocol. A 40-μL reaction included 35 μg of pupal RNA, 1 μL of RNase inhibitor (Promega), 2.5 mM $MgCl_2$, 0.2 mM dNTP, 30 pmole Qt primer and 1 μL of GoScript (Promega). Two separate reactions were incubated in 42 °C for 1 h, followed by 50 °C for 20 min. The two reactions were pooled and cDNA was purified using Qiaquick kit (Qiagen) with final elution of 50 μL.

Table 1
PCR Primers used in this study.

Name	Sequence (5′- to 3′)
Qt	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTT
Q0	CCAGTGAGCAGAGTGACG
Q1	GAGGACTCGAGCTCAAGCTT
dg-rpr-f1	ATG GCI[a] GTI GCI TTY TAY AT (MAVAFYI[b])
dg-rpr-f2	TTY TAY[a] ATI CCI GAY CAR GC (FYIPDQA[b])
UAS-f	GAATACAAGAAGAGAACTCTG
UAS-r	GTCCAATTATGTCACACCAC
F1	CACCTCTACAAAGTCCTGTCAC
F2	CTGCGAAGCTTTGCATCGGT
F3	GACACTCGAAAACGAACAGCGA
R1	CGTCTTGTCGATGTTCTTGGAG
R2	GTCACCACCATTGTAGCATGGA
R3	CGCTGTTCGTTTTCGAGTGTC
5′end	CGTTCAGTCTTTCAGTCGTAGCT
3′end	GAATTCTTCCATAAAACGGAAACATTTTCGCT
5′ORF	GCCAGATCTAAGTC **ATG** GCT GTG CCT T (*Bgl* II site underlined, start codon in boldface)
3′ORF	GCCTCTAGATTCTTATAG **TCA** TCG TGC (*Xba* I site underlined, stop codon in boldface)
ΔIBM-f	GCCAGATCTAAGTC **ATG** AAT CAG GAA GAT GGA GAT
ΔMTS-r	GCCTCTAGATTCTTATAG **TCA** ACA TGG ATC CTT AGC CTT
RTF1	CCAAGAGATAGAAGCACCCCATTG
RTF2	GCGAACATGAGACTAGTTGGGATG
RTF3	TTTGAAACTAAAGTGCTCATAGAAC
RTR1	GCCTTTGAATTCTTGCAACCTCTCC
RTR2	GATATCCACAACCCATGGCAAATACG
TUF1	GCTTACAAGGTCTCCAATTGACTCATTC
TUR1	GTTCGACGACTGTGTCTGATACTTTTGG
Int1R	TAGAAGATTTAGAGTGCCAAAAC
Int2R	GAGCATTACCCACAAACTTTTC
Int3R	GTGAAGAGGTTAGAGGTTCTG

[a] I indicates inosine.
[b] Amino acid residues corresponding to codons.

PCR (3R-I) included GoTaq premix (Promega), 2 μL of dg-rpr-f1 primer (10 μM), 4 μL of Q0 primer (2 μM) and 1 μL of the purified cDNA in a total of 30-μL reaction. After initial denaturation at 94 °C for 2 min, 30 cyclic reactions (94 °C, 40 s → 47 °C, 40 s → 72 °C, 80 s) followed by a final extension (72 °C, 5 min) were performed. This PCR was diluted 1:20 in water, and then 1 μL of it was added to a nested PCR (3R-II, 40 μL), which included 2.5 μL dg-rpr-f2 (10 μM) and 4 μL Q1 primer (2 μM). And then PCR was run using the following parameters: 94 °C, 2 min → (94 °C, 40 s → 50 °C, 40 s → 72 °C, 80 s: 32 cycles) → 72 °C, 7 min. Control PCRs including only one primer were run in parallel. Agarose gel electrophoresis revealed specific but faint PCR product at ~800-bp position. To amplify this product, 3R-II was diluted 1:20 in water, 1 μL of which was used for the PCR that was done the same way as 3R-II. The amplified product was gel-purified using Qiaquick purification kit, cloned into the *pGEMTeasy* vector (Promega) and then sequenced.

2.3. 5′-RACE

According to the 3′ RACE sequence result, two gene-specific reverse primers (R1 and R2) were designed. Thirty picomole of R1 primer was used to make cDNA in the same manner as cDNA synthesis for 3′ RACE. The reverse transcription reaction was purified by using Qiaquick kit (30 μL final elution). To this, the following reagents were added for poly-A tailing reaction (60 μL): dATP, Terminal deoxynucleotide transferase (Promega), and Qt primer, as described previously (Sha et al., 2012). The reaction was incubated at 37 °C for 6 min, and then terminated at 65 °C for 5 min. The resulting R1-primed and poly-A-tailed cDNA was purified using Qiaquick kit (50 μL final elution).

First PCR (5R-I) was conducted with Qt and R1 primer, as described for 3R-II. It was then diluted 1:20 in water, and 1 μL of this was added to 50 μL of a nested PCR (5R-II) including Q1 and R2 primer. Three distinct PCR products were identified between 500-bp and 800-bp markers.