Developmental Biology 346 (2010) 150–160

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Evolution of Developmental Control Mechanism

The hormonal pathway controlling cell death during metamorphosis in a hemimetabolous insect

Daniel Mané-Padrós [1], Josefa Cruz, Lluisa Vilaplana [2], Claudia Nieva, Enric Ureña, Xavier Bellés, David Martín *

Institute of Evolutionary Biology (CSIC-UPF), Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain

ARTICLE INFO

Article history:
Received for publication 2 March 2010
Revised 28 June 2010
Accepted 9 July 2010
Available online 16 July 2010

Keywords:
Programmed cell death
20-Hydroxyecdysone
Juvenile hormone
Prothoracic gland
Hemimetabolous insect
Blattella germanica

ABSTRACT

Metamorphosis in holometabolous insects is mainly based on the destruction of larval tissues. Intensive research in *Drosophila melanogaster*, a model of holometabolan metamorphosis, has shown that the steroid hormone 20-hydroxyecdysone (20E) signals cell death of larval tissues during metamorphosis. However, *D. melanogaster* shows a highly derived type of development and the mechanisms regulating apoptosis may not be representative in the insect class context. Unfortunately, no functional studies have been carried out to address whether the mechanisms controlling cell death are present in more basal hemimetabolous species. To address this, we have analyzed the apoptosis of the prothoracic gland of the cockroach *Blattella germanica*, which undergoes stage-specific degeneration just after the imaginal molt. Here, we first show that *B. germanica* has two inhibitor of apoptosis (IAP) proteins and that one of them, BgIAP1, is continuously required to ensure tissue viability, including that of the prothoracic gland, during nymphal development. Moreover, we demonstrate that the degeneration of the prothoracic gland is controlled by a complex 20E-triggered hierarchy of nuclear receptors converging in the strong activation of the death-inducer Fushi tarazu-factor 1 (BgFTZ-F1) during the nymphal–adult transition. Finally, we have also shown that prothoracic gland degeneration is effectively prevented by the presence of juvenile hormone (JH). Given the relevance of cell death in the metamorphic process, the characterization of the molecular mechanisms regulating apoptosis in hemimetabolous insects would allow to help elucidate how metamorphosis has evolved from less to more derived insect species.

Introduction

Holometabolan metamorphosis is a key innovation in insect development, which involves the stage-specific destruction of a number of tissues at the end of the larval growth and the formation of new adult structures. In this context, the selection of highly regulated mechanisms to control developmentally induced cell death has been a key associated event. In *Drosophila melanogaster*, a model of holometabolan metamorphosis, intensive research has revealed the steroid hormone 20-hydroxyecdysone (20E) as the main regulator of the destruction and elimination of larval tissues. In fact, two pulses of 20E, one at the end of the larval instar and the second approximately 10 h after the formation of the puparium, are responsible of the destruction of the larval midgut and the salivary glands, respectively (Yin and Thummel, 2005).

From a molecular point of view, a genetic cascade of transcription factors mediates the apoptotic effect of 20E. Upon binding to its heterodimeric receptor composed by the two nuclear receptors Ecdysone receptor (EcR) and Ultraspiracle (Usp), 20E activates the transcription of a set of genes including *broad* (zinc finger transcription factor), *E74* (ETS-domain transcription factor), *E93* (helix-turn-helix DNA binding protein) and a number of nuclear hormone receptors, such as E75, HR3 and FTZ-F1 which act as a competence factor for genetic responses to the prepupal–pupal ecdysteroid pulse (Baehrecke and Thummel, 1995; Bialecki et al., 2002; Broadus et al., 1999; Burtis et al., 1990; DiBello et al., 1991; Lam et al., 1999). In turn, these genes induce the expression of several secondary late genes including death inducers *reaper* (*rpr*) and *head involution defective* (*hid*), as well as the main executers of apoptotic death, the initiator caspase *Dronc*, the effector caspase *Drice* and the adaptor protein *Dark* (Thummel, 1996; Lee et al., 2000; Jiang et al., 2000; Cakouros et al., 2002). Interestingly, before the pupal stage, these caspases are kept as inactive proteins by the activity of the *Drosophila* inhibitor of apoptosis 1 (DIAP1). DIAP1 is defined by the presence of two Baculovirus IAP Repeat (BIR) domains that function as protein interaction modules that bind to caspases and prevent their activity (Orme and Meier, 2009). Rpr and Hid proteins induce cell death by

* Corresponding author. Fax: +34 932211011.
E-mail address: david.martin@ibe.upf-csic.es (D. Martín).
[1] Present address: Department of Cell Biology & Neuroscience, University of California, Riverside, CA 92521, USA.
[2] Present address: Center for Research in Agricultural Genomics (CRAG), Consortium CSIC-IRTA-UAB, Spain.

0012-1606/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2010.07.012

interacting with DIAP1, thus impairing its inhibitory activity (Martin, 2002). In addition to 20E, another hormone involved in cell death is the sesquiterpenoid juvenile hormone (JH). Although the molecular mechanism by which JH operates remains basically unknown (Riddiford, 2008), it has been shown that this hormone prevents degeneration of the midgut in the dipteran *Aedes aegypti* (Wu et al., 2006; Parthasarathy and Palli, 2007a) and the lepidopteran *Heliothis virescens* (Parthasarathy and Palli, 2007b), and also antagonizes the 20E-induced apoptosis of the larval fat body of *D. melanogaster* by counteracting the activity of two transcription factors of the bHLH-PAS family that play crucial roles in JH action, Methoprene-tolerant and its paralog Germ-cell expressed (Liu et al., 2009).

It is worth noting, however, that *D. melanogaster* shows a highly derived type of development, so the mechanisms regulating apoptosis may be poorly representative in the insect class context. Unfortunately, no functional studies have been carried out to address whether these mechanisms are present in more basal hemimetabolous species. Unlike the holometabolous, hemimetabolous species hatch from the egg as first instar nymphs that resemble miniature adults. In these insects, growth and maturation occur simultaneously throughout sequential nymphal stages until the imaginal molt. Nevertheless, the absence of an intermediate pupal stage in hemimetabolous insects does not imply the lack of stage-specific cell death. In this sense, using the German cockroach, *Blattella germanica*, as a hemimetabolan model, we have shown that the prothoracic gland, which is responsible for the synthesis of ecdysteroids, undergoes a precise developmentally controlled degeneration just after the imaginal molt (Romañá et al., 1995; Cruz et al., 2006; Mané-Padrós et al., 2008). Since winged adult insects, as *B. germanica*, do not undergo further molting, the elimination of the ecdysteroid source at the onset of the adult stage is crucial to allow normal adult development. Thus, the degeneration of the prothoracic gland in a hemimetabolous species is a good model to analyze a less derived developmentally regulated apoptotic event during the post-embryonic development of an insect. Importantly, the characterization of the molecular mechanisms that regulate cell death in hemimetabolous insects would allow to understand how metamorphosis has evolved from less to more derived insect species.

In this study, by using an RNAi in vivo approach, we have functionally characterized activator as well as inhibitor proteins that coordinate the degeneration of the prothoracic gland during the onset of the adult stage of *B. germanica*. First, we have shown that this insect has two IAP proteins and that one of them, BgIAP1, is constantly required to block premature apoptosis during nymphal development. We have also demonstrated that the degeneration of the prothoracic gland depends on a complex 20E-triggered hierarchy of nuclear receptors that converges on the precise activation of the orphan nuclear receptor Fushi tarazu-factor 1 (BgFTZ-F1), which is responsible of the degeneration of the prothoracic gland at the onset of adult development. Finally, we have shown that JH plays a critical role in preventing prothoracic gland degeneration during nymphal development of *B. germanica*.

Materials and methods

Insects

Specimens of *B. germanica* were obtained from a colony reared in the dark at 30 ± 1 °C and 60–70% r.h. All dissections and tissue sampling were carried out on carbon dioxide-anaesthetized specimens.

Cloning of BgIAP1 and BgIAP2

A 765 bp clone corresponding to BgIAP1 was obtained from a suppression subtractive hybridization library that was carried out using the PCR-selected cDNA Subtraction Kit (Clontech), following the manufacturer's protocols. The tester library was prepared with 1 μg of polyA$^+$ mRNA from UM-BGE-1 embryonic cells from *B. germanica* treated with 20E during 10 h. The driver library was prepared with the same amount of polyA$^+$ mRNA from untreated UM-BGE-1 cells. To complete the sequence of the BIR domains, 5′RACE (5′RACE System Version 2.0; Invitrogen) was carried out using as reverse primer (BgIAP1R1), 5′-AACGTTGATGATCCCTGAATG-3′ and a nested reverse primer (BgIAP1R2), 5′-GGACATCTAACTACATCACTTCGA-3′.

B. germanica IAP2 cDNA sequence was isolated from a cDNA library prepared by M.D. Piulachs with an ovary pool of adult *B. germanica* including samples of each day of the first gonadotrophic cycle. To construct this library, total RNA was extracted using GenElute Mammalian Total RNA kit (Sigma). Subsequently, polyA$^+$ mRNA was obtained taking advantage of the Dynabeads Oligo (dT) technology (Dynal Biotech ASA). cDNA synthesis was performed with the lambda ZAPII cDNA Synthesis Kit (Stratagene) according to the manufacturer's protocol. Among the sequences obtained after massive sequencing, a fragment of 1056 bp, corresponding to the putative IAP2, was obtained (M.D. Piulachs and P. Irles, unpublished results). This fragment was extended to the 3′ end by a 3′RACE approach (Invitrogen) using the forward primer: 5′-CAGAGCTC-GACTGTGAGAGACTTA-3′. The product obtained was subcloned into the pSTBlue-1 vector (Novagen) and sequenced. Amino acid sequence comparisons indicated that the protein showed the characteristic domains of this family, precisely 3 BIR domains and 1 RING finger.

RNA interference

RNAi in vivo in nymphs of *B. germanica* was performed as described in Martín et al. (2006) and Cruz et al. (2007). The primers used to generate templates via PCR for transcription of the dsRNAs are described in Supplemental Table 1. A volume of 1 μl of each dsRNA solution (1 μg/μl) was injected into the abdomen of newly emerged female nymphs. In case of coinjection of two dsRNAs, 1 μl of each solution was applied in a single injection of 2 μl.

Treatments with methoprene in vivo

Newly ecdysed last instar nymphs were topically treated with 1 μg methoprene (isopropyl (*E,E*)-(*RS*)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate) per specimen in 1 μl of acetone. Controls received the same volume of solvent.

Quantification of haemolymph ecdysteroids

Haemolymph ecdysteroids were quantified by ELISA following the procedure described by Porcheron et al. (1989), and adapted to *B. germanica* by Pascual et al. (1992) and Romañá et al. (1995). 20E (Sigma) and 20E-acetylcholinesterase (Cayman) were used as standard and enzymatic tracer, respectively. The antiserum (Cayman Chemical) was used at a dilution of 1:50,000. Absorbances were read at 450 nm, using a Multiscan Plus II Spectrophotometer (Labsystems). The ecdysteroid antiserum used has the same affinity for ecdysone and 20E (Porcheron et al., 1989), but since the standard curve was obtained with the later compound, results are expressed as 20E equivalents.

Microscopy, histological analysis and detection of cell death

All dissections of larval and adult tissues were carried out in Ringer's saline. Prothoracic glands were fixed in 4% paraformaldehyde and permeabilised in PBS–0.2% tween (PBT), then incubated for 10 min in 1 μg/ml DAPI in PBT. After two washes with PBT, the tissues were mounted in Mowiol 4-88 (Calbiochem). All samples were examined with a Zeiss Axiophot microscope, and images were subsequently processed using Adobe Photoshop. To detect cell death, TUNEL assays were