Biochimica et Biophysica Acta 1830 (2013) 2178–2187

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Broad-complex functions in postembryonic development of the cockroach *Blattella germanica* shed new light on the evolution of insect metamorphosis

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ARTICLE INFO

Article history:
Received 1 August 2012
Received in revised form 27 September 2012
Accepted 28 September 2012
Available online 3 October 2012

Keywords:
Insect metamorphosis
Juvenile hormone
Ecdysone
Evolution of holometaboly
Drosophila
Tribolium

ABSTRACT

Background: Insect metamorphosis proceeds in two modes: hemimetaboly, gradual change along the life cycle; and holometaboly, abrupt change from larvae to adult mediated by a pupal stage. Both are regulated by 20-hydroxyecdysone (20E), which promotes molts, and juvenile hormone (JH), which represses adult morphogenesis. Expression of Broad-complex (BR-C) is induced by 20E and modulated by JH. In holometabolous species, like *Drosophila melanogaster*, BR-C expression is inhibited by JH in young larvae and enhanced in mature larvae, when JH declines and BR-C expression specifies the pupal stage.
Methods: Using *Blattella germanica* as a basal hemimetabolous model, we determined the patterns of expression of BR-C mRNAs using quantitative RT-PCR, and we studied the functions of BR-C factors using RNA interference approaches.
Results: We found that BR-C expression is enhanced by JH and correlates with JH hemolymph concentration. BR-C factors appear to be involved in cell division and wing pad growth, as well as wing vein patterning.
Conclusions: In *B. germanica*, expression of BR-C is enhanced by JH, and BR-C factors appear to promote wing growth to reach the right size, form and patterning, which contrast with the endocrine regulation and complex functions observed in holometabolous species.
General significance: Our results shed new light to the evolution from hemimetaboly to holometaboly regarding BR-C, whose regulation and functions were affected by two innovations: 1) a shift in JH action on BR-C expression during young stages, from stimulatory to inhibitory, and 2) an expansion of functions, from regulating wing development, to determining pupal morphogenesis.

1. Introduction

The origin and evolution of insect metamorphosis poses one of the most enigmatic conundrums in evolutionary biology. In his "On the Origin of Species", Charles Darwin already complained about the difficulty of integrating insect metamorphosis (due to the striking difference between the morphologies and life styles of larvae and adults of the same species) into his theory of species evolution by natural selection [1]. However, it is clear that insect metamorphosis has been a key innovation in insect evolution as most of the present biodiversity on Earth is composed of metamorphosing insects, with approximately 1 million species described, and 10–30 million still to be discovered [2,3].

The first systematic studies on insect metamorphosis were carried out by Renaissance entomologists, who established that post-embryonic changes are most spectacular in insects like butterflies, beetles and flies, which undergo a dramatic morphological transformation from larva to pupa and adult, a phenomenon now known as holometaboly. Other insects, such as locusts and cockroaches, also metamorphose from the last nymphal instar to adult, although the change of form is not as radical given that the nymphs are similar to the adults. However, they undergo qualitative metamorphic changes, such as formation of mature wings and external genitalia in a type of metamorphosis known as hemimetaboly [4,5]. Metamorphosis evolved from hemimetaboly to holometaboly, and the latter innovation was most successful because more than 80% of present insects are holometabolous species (including the "big four" orders: Lepidoptera, Coleoptera, Diptera and Hymenoptera) [2,3]. Therefore, explaining the evolutionary transition from hemimetaboly to holometaboly may give a new look to explain how this amazing biodiversity originated, and the study of the processes regulating metamorphosis shall surely provide important clues for such a goal [6].

Insect metamorphosis is regulated by two hormones, the molting hormone, which promotes molting, and the juvenile hormone (JH), which represses metamorphosis and, thus determines the molt type: to an immature stage when it is present, or to the adult when it is absent [4,6,7]. Although the molecular action of JH is still poorly understood [8], we know that an important transducer of the JH signal is Methoprene tolerant (Met), a transcription factor that was discovered in *Drosophila*

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http://dx.doi.org/10.1016/j.bbagen.2012.09.025

melanogaster and that plays an important role in JH reception [9]. Key functional evidence that Met is required for the repressor action of JH on metamorphosis was obtained from the beetle *Tribolium castaneum*, a basal holometabolous insect where depletion of Met expression induced larvae to undergo precocious metamorphosis [10,11]. More recently, the function of Met as an early JH transducer has been demonstrated in the hemimetabolous species *Pyrrhocoris apterus* [12], which established the first regularity in the signaling pathway of JH in hemimetabolous and holometabolous insects. Another important element in JH transduction in relation to metamorphosis is the transcription factor Krüppel homolog 1 (Kr-h1), whose antimetamorphic action was firstly demonstrated in *D. melanogaster* [13] and *T. castaneum* [14]. More recently, the role of Kr-h1 as a transducer of the JH signal has been reported in three hemimetabolous insects: the cockroach *Blattella germanica* [15] and the bugs *P. apterus* and *Rhodnius prolixus* [12]. RNAi studies in these species have shown that Kr-h1 represses metamorphosis and that it acts downstream of Met in the JH signaling pathway. Kr-h1 therefore appears to be the more distal transcription factor in the JH signaling cascade whose role as mediator of the antimetamorphic action of JH has been conserved from cockroaches to flies. The next challenge is to unveil the factor(s) specifying the adult stage that are repressed by Kr-h1.

Concerning the molecular action of molting hormones, the effect of 20-hydroxyecdysone (20E) is also mediated by a cascade of transcription factors that starts upon its binding to the heterodimeric receptor composed of the ecdysone receptor and the ultraspiracle, which belong to the nuclear receptor superfamily. This activates expression of a hierarchy of transcription factors generally belonging to the same superfamily, like E75, E78, HR3, HR4 and FTZ-F1, which regulate the genes that underlie the cellular changes associated to molting and metamorphosis [16,17]. Most of the information available on this cascade refers to *D. melanogaster* [18,19], but there are a good deal of data from hemimetabolous species, especially from the cockroach *B. germanica*. Factors involved in 20E signaling in *B. germanica* are generally the same as in *D. melanogaster*, although the functions of some of them and their epistatic relationships may differ with respect to those observed in the fly [20–22].

Among the most interesting 20E-dependent factors are the products of the Broad-complex (BR-C) gene, whose functions may have radically diverged in hemimetabolous and holometabolous species. BR-C encodes a group of C2H2 zinc-finger transcription factors [23,24] that, in holometabolous species, like the dipteran *D. melanogaster*, the lepidopterans *Manduca sexta* and *Bombyx mori*, and the coleopteran *T. castaneum*, are expressed in the final larval stage, and this transient expression is essential for the successful formation of the pupae [11,25–28]. Experiments carried out on the hemipterans *Oncopeltus fasciatus* [29] and *P. apterus* [12], which are phylogenetically distal hemimetabolous species, suggested that BR-C transcription factors only regulate gradual wing bud growth. This specific role, which is radically different from the morphogenetic functions involved in pupae formation in holometabolous species, prompted us to undertake a detailed functional study of BR-C in *B. germanica*, a basal polyneopteran insect representing a poorly modified hemimetabolous species [4]. In *B. germanica*, the BR-C gene encodes six zinc-finger isoforms (BR-C Z1 to Z6), which play important roles in embryogenesis [30]. The present work, based on functional studies in post-embryonic development, reveals ancestral functions of BR-C transcription factors related to cell division and of wing pad growth, as well as to wing vein patterning, and provides new clues that illuminate the evolution of insect metamorphosis.

2. Materials and methods

2.1. Insects

B. germanica specimens used in the experiments were obtained from a colony reared in the dark at 30 ± 1 °C and 60–70% r.h. They were carbon dioxide-anesthetized prior to dissections and tissue sampling.

2.2. RNA extraction and retrotranscription to cDNA

All RNA extractions were carried out with the Gen Elute Mammalian Total RNA kit (Sigma-Aldrich, Madrid, Spain). An amount of 400 ng from each RNA extraction was treated with DNase (Promega, Madison, WI, USA) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers (Promega). RNA quantity and quality were estimated by spectrophotometric absorption at 260 nm in a Nanodrop Spectrophotometer ND-1000® (NanoDrop Technologies, Wilmington, DE, USA).

2.3. Determination of mRNA levels with quantitative real-time PCR

Quantitative real time PCR (qRT-PCR) reactions were carried out in triplicate in an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Madrid, Spain), using SYBR®Green (Power SYBR® Green PCR Master Mix; Applied Biosystems, Madrid, Spain). A control without a template was included in all batches. The primers used to detect all isoforms simultaneously or to detect each isoform specifically are described in Table S1 (see Supplementary data). The efficiency of each primer set was first validated by constructing a standard curve through four serial dilutions. mRNA levels were calculated relative to BgActin-5c (accession number: AJ862721) expression using the Bio-Rad iQ5 Standard Edition Optical System Software (version 2.0). The primers used to quantify BgActin-5c are indicated in Table S2 (see Supplementary data). We followed a method based in Ct (threshold-cycle) according to the Pfaffl mathematical model [31], simplifying to $2^{\Delta\Delta Ct}$ because the calculated efficiency values for studied genes and BgActin-5c amplicons were always within the range of 95 to 100%; therefore, no correction for efficiency was used in further calculations. Results are given as copies of mRNA per 1000 copies of BgActin-5c mRNA.

2.4. Treatments with juvenile hormone III in vivo

To study the effect of JH upon BR-C expression, JH III, which is the native JH of *B. germanica* [32,33], was applied topically to freshly emerged last instar nymphs at a dose of 20 μg per specimen in 1 μl of acetone. We used JH III from Sigma-Aldrich, which is a mixture of isomers containing about 50% of the biologically active (10R)-JH III. Thus, the active dose applied would be around 10 μg per specimen, which is an efficient dose to impair metamorphosis [34]. Controls received 1 μl of acetone.

2.5. RNA interference

B. germanica is very sensitive to RNA interference (RNAi) in vivo [35]. Detailed procedures for dsRNA preparation and RNAi experiments were as described previously [22,36,37]. Concerning BR-C, dsRNAs were prepared to deplete all isoforms simultaneously (dsBrCore) or specific isoforms BR-C Z1 to BR-C Z6 (dsBrZ1 to dsBrZ6). The primers used to generate templates with PCR for transcription of these dsRNAs are described in Table S3 (see Supplementary data). The fragments were amplified by PCR and cloned into the pSTBlue-1 vector (Novagen, Madrid, Spain). In all cases, we used a 307 bp sequence from *Autographa californica* nucleopolyhydrosis virus (accession number: K01149, from nucleotides 370 to 676) as control dsRNA (dsMock). A volume of 1 μl of each dsRNA solution (3 μg/μl) was injected into the abdomen of specimens at chosen ages and stages. The control specimens were treated with the same dose and volume of dsMock. RNAi of Kr-h1was carried out as recently reported [15].