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Nuclear receptor HR4 plays an essential role in the ecdysteroid-triggered gene cascade in the development of the hemimetabolous insect *Blattella germanica*

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ABSTRACT

Despite the differences in the developmental strategies between hemimetabolous and holometabolous insects, a common feature between both types of development is that periodic pulses of the steroid hormone 20-hydroxyecdysone (20E) dictate each developmental transition. Although the molecular action of 20E has been extensively studied in holometabolous insects, data on hemimetabolous is scarce. To address this, we have used the German cockroach *Blattella germanica* to show that 20E signals through a transcriptional cascade of the nuclear hormone receptor-encoding genes *BgE75*, *BgHR3* and *BgFTZ-F1*. Here, we report the isolation and functional characterization of BgHR4, another nuclear receptor involved in this cascade. Expression studies along with tissue incubations and RNAi experiments show that cross-regulation between BgE75 and BgHR3 directs the expression of *BgHR4*. Finally, we have also shown that *BgHR4* is an essential gene required for successfully completing nymphal–nymphal and nymphal–adult transitions, by allowing the appropriate delay in the induction of *BgFTZ-F1*.

1. Introduction

In hemimetabolous insects, growth and maturation occur simultaneously throughout successive nymphal stages until the imaginal molt. This type of development contrasts with that of holometabolous insects, in which an intermediate pupal stage occurs between the juvenile (larval) and adult stages. In holometabolous insects, growth is restricted to larval development, whereas maturation takes place during metamorphosis in the pupal stage. A common feature in both types of development, however, is the central regulatory role exerted by the ecdysteroid hormone 20-hydroxyecdysone (20E). Periodic pulses of 20E trigger nymphal/larval molts and, in holometabolous insects, a pulse of 20E at the end of the last larval instar signals the onset of pupation where it controls the destruction of larval tissues and the formation of the adult structures during metamorphosis (Riddiford, 1993; Thummel, 1995, 2001).

Although there is a detailed understanding of the molecular mechanisms by which 20E regulates the metamorphic process in holometabolous insects, especially in *Drosophila melanogaster* (Thummel, 1996; Riddiford et al., 2000, 2003; King-Jones and Thummel, 2005), little is known about how this hormone operates during the development of hemimetabolous species. Given that holometabolous metamorphosis arose from hemimetabolous ancestors (Sehnal et al., 1996; Truman and Riddiford, 1999; Belles, 2011), it would be interesting to study whether the regulatory mechanisms of 20E action in holometabolous insects are also present in more primitive hemimetabolous insects. We have been using the German cockroach, *Blattella germanica*, to characterize the 20E-triggered hierarchy of transcription factors that responds to, and transduces, the hormonal signal in hemimetabolous insects. In this cockroach, 20E acts upon binding to its heterodimeric receptor formed by the Ecdysone receptor (BgEcR-A) and the retinoid X receptor/ultraspiracle (BgRXR), both expressed in a housekeeping-like pattern throughout nymphal development (Cruz et al., 2006; Martín et al., 2006). In response to 20E binding to BgEcR–BgRXR, several isoforms encoded by three nuclear receptor genes (*BgE75*, *BgHR3* and *BgFTZ-F1*) are sequentially activated and repressed during the second part of the last nymphal instar (Fig. 1) (Cruz et al., 2007; Mané-Padrós et al., 2008). This cascade of nuclear receptors, which is present in most tissues of *B. germanica*, not only controls ecdysteroid biosynthesis and molting during each nymphal instar but also metamorphic-associated processes, such as cell proliferation in wings and in follicular epithelium, and the programmed cell death of the prothoracic gland (Cruz et al., 2006, 2007, 2008; Martín et al., 2006; Mané-Padrós et al., 2008, 2010).

To further complete the characterization of this genetic cascade in *B. germanica*, we have now cloned and characterized a new 20E-dependent nuclear receptor that plays a key role in the regulation of such hierarchy, namely HR4. This nuclear receptor, whose

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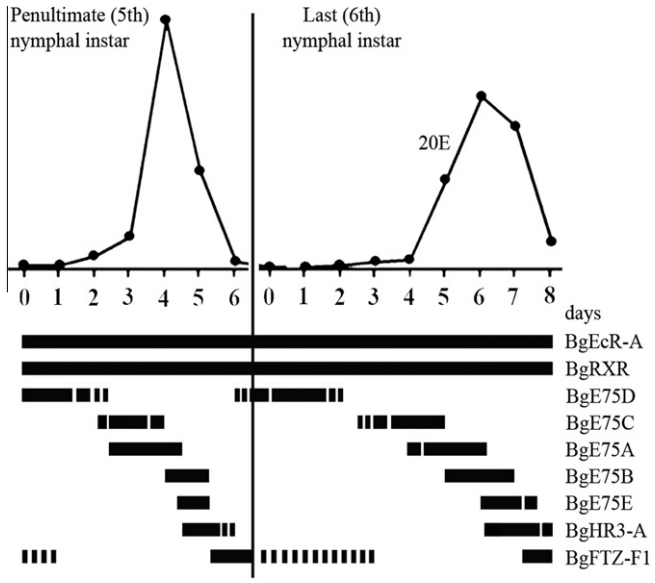

Fig. 1. Circulating ecdysteroid levels and mRNA expression of 20-hydroxyecdysone-dependent nuclear receptors during the last two nymphal instars of *Blattella germanica*. The ecdysteroid (20E) levels are redrawn from Cruz et al. (2003). The diagrams of mRNA expression are based on Cruz et al. (2006, 2007, 2008), Maestro et al. (2005) and Mané-Padrós et al. (2008).

corresponding mammalian ortholog is the transcriptional repressor Germ Cell Nuclear Factor (GCNF) (Fuhrmann et al., 2001; Lan et al., 2002; Sato et al., 2006), has been previously characterized only in holometabolous species, namely the coleopterans *Tenebrio molitor* (Mouillet et al., 1999) and *Tribolium castaneum* (Tan and Palli, 2008), the lepidopterans *Manduca sexta* (Weller et al., 2001) and *Bombyx mori* (Charles et al., 1999) and the dipteran *D. melanogaster* (King-Jones et al., 2005), although loss of function analysis has been carried out only in *T. castaneum* and *D. melanogaster*. In *T. castaneum*, TcHR4 is required for the pupal molt and for successful vitellogenesis and oogenesis during the adult stage (Tan and Palli, 2008; Xu et al., 2010). In *D. melanogaster*, DHR4 exerts a central role coordinating growth and maturation during the last larval instar (King-Jones et al., 2005).

Here, we report the cloning and functional characterization of the HR4 homolog of *B. germanica*, named BgHR4. First, we have examined its developmental expression during embryo development and throughout nymphal stages, as well as the 20E-responsiveness of the gene. Furthermore, by using RNAi in vivo, we have showed that *BgHR4* is a vital gene required for normal development during all the nymphal stages of *B. germanica*. Importantly, we have shown that BgHR4 plays a key role in the 20E-responsive transcriptional cascade by down-regulating the levels of three BgE75 isoforms at the end of the nymphal instar, thus allowing the appropriate delay in the transcriptional up-regulation of the nuclear receptor *BgFTZ-F1*, a master regulator of *B. germanica* development.

2. Materials and methods

2.1. Insects

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

2.2. Cloning of BgHR4 cDNA

The *B. germanica* HR4 homologue was obtained by PCR using cDNA template from 20E-treated UM-BGE-1 embryonic cells from *B. germanica*, following the methodology previously described (Maestro et al., 2005; Cruz et al., 2006). Degenerate primers for BgHR4 amplification were, forward (BgHR4-F1): 5′-ATGATVTGYG ARGAYAARGC-3′, and reverse (BgHR4-R1): 5′-TGYTCDATRCAYTT YTTRAA-3′. The amplified fragment (189 bp) was subcloned into the pSTBlue-1 vector (Novagen) and sequenced. This was followed by 5′ and 3′ RACE (5′- and 3′-RACE System Version 2.0; Invitrogen) to extend the sequence. For 5′RACE, reverse primers were (BgHR4-R2): 5′-TACAAGTTGTAAACAGCTCCAGAGTT-3′, the nested (BgHR4-R3): 5′-CGTAGTGCAGACCTGTAGCCTTAT-3′ and the nested (BgHR 4-R4): 5′-TTGCGACCAGATCATCGTCCTTACA-3′. For 3′RACE, forward primer was (BgHR4-F2): 5′-ACCACAAGTCTGATCTCTCAGACAA-3′ and nested (BgHR4-F3): 5′-CACACAAGATTATTAACGCACAAGTG-3′. All PCR products were subcloned into the pSTBlue-1 vector (Novagen, Madison, WI, USA) and sequenced.

2.3. RT-PCR/Southern blot analyses

RT-PCR followed by Southern blotting with specific probes was used to establish the expression patterns of BgHR4. The RNA was obtained from different tissues, and synthesis of cDNA was carried out as previously described (Cruz et al., 2003). Primers used to amplify the different target genes are detailed in Supplemental Table S1. cDNA samples were subjected to PCR with a number of cycles within the linear range of amplification for each transcript depending on the tissue and physiological stage, as previously described (Cruz et al., 2006, 2007). cDNA probes for Southern blot analyses were generated by PCR with the same primer pairs using plasmid DNAs containing the corresponding cDNA clones as templates. The probes were labeled with fluorescein, using the Gene Images random prime-labeling module (Amersham Biosciences, Barcelona, Spain). RT-PCR followed by Southern blotting of total RNA without reverse transcription did not result in amplification, indicating that there was no genomic contamination.

2.4. Incubation of epidermis/fat body in vitro

Abdominal tergites with epidermis and adhering fat body tissue were dissected from sixth instar female nymphs and incubated in 1 ml of Grace's medium, with L-glutamine and without insect hemolymph (Sigma, Madrid, Spain) at 30 °C in the dark as described (Cruz et al., 2006).

2.5. RNA interference

RNAi in vivo in nymphs of *B. germanica* was performed as previously described (Martín et al., 2006; Cruz et al., 2007). The primers used to generate templates via PCR for transcription of the dsRNAs are detailed in Supplemental Table S2. Control dsRNA consisted of a non-coding sequence from the pSTBlue-1 vector (dsMock) (Cruz et al., 2006). A volume of 1 μl of dsRNA solution (1 μg/μl) was injected into the abdomen of newly emerged fifth or sixth instar female nymphs. In case of coinjection of two dsRNAs, a single injection of 2 μl, consisted of 1 μl of each solution, was applied.

2.6. Microscopy, histological analysis and quantification of hemolymph ecdysteroids

All dissections were carried out in Ringer's saline (9 g/l NaCl, 0.2 g/l KCl, 0.2 g/l $NaHCO_3$, 0.2 g/l $CaCl_2$). Mouthparts and tracheae were directly immersed in 50% glycerol and examined