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.

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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
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 experiments 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 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'-ATGATCTGTYG ARGAYAARGC-3', and reverse (BgHR4-R1): 5'-TGTYCDATRCAYTT 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'-TACAAGTTGTAAACGCTCAAGT'T-3', the nested (BgHR4-R3): 5'-CGTAGTCGACACTGTTAGCCAT-3' and the nested (BgHR4-R4): 5'-TGCGACAGACATCCTGCTTACA-3'. For 3' RACE, forward primer was (BgHR4-F2): 5'-ACCAGAATCTGCTTCACAGA-3' and nested (BgHR4-F3): 5'-CACAGAATATTAAAGCACAAGT-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). RT-PCR was performed as previously described (Cruz et al., 2003). 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 S2. 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.

2.5. 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 NaHCO3, 0.2 g/l CaCl2). Mouthparts and tracheae were directly immersed in 50% glycerol and examined
microscopically. To examine the cuticle layers, a portion of an abdominal sternite was fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Cuticle sections (6 μm) were stained with toluidine blue. All samples were examined with a Zeiss Axioskop microscope. Hemolymph ecdysteroids were quantified by ELISA as previously described (Romahá et al., 1995). 20E (Sigma) and 20E-acyethylcholinesterase (Cayman Chemical, Ann Arbor, MI, USA) were used as a standard and an enzymatic tracer, respectively. The antisemur (Cayman Chemical) was used at a dilution of 1:50,000. Absorrences were read at 450 nm, using a Multiscan Plus II Spectrophotometer (Labsystems, Madrid, Spain). The ecdysteroid antisemur 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.

3. Results

3.1. Cloning and characterization of BgHR4

Cloning of BgHR4 cDNA was accomplished by a RT-PCR approach using degenerate primers designed on the basis of conserved motifs of the DNA binding domain (DBD) of available insect HR4 sequences. Using cDNA from 20E-treated B. germanica UM-BGE-1 cells as a template, a 189 bp PCR fragment was obtained, and its sequence was highly similar to insect HR4 sequences. 3’- and 5’-RACE experiments allowed us to extend the sequence in both directions (GenBank accession number JF758869). The protein corresponding to the cDNA obtained showed the domain organization characteristic of a nuclear hormone receptor: a ligand-independent A/B activation domain, followed by the two zinc fingered DBD (C domain) with and adjacent stretch of 32 highly conserved amino acids known as the carboxy-terminal extension (CTE), a hinge region (D domain) and the ligand binding domain (LBD; E domain), which did not contain the putative ligand-independent activation motif, AF-2, and finally a very short F domain (14 amino acids).

The comparison with other HR4 and GCNF sequences (Fig. 2) revealed that the most conserved domains are the DBD (100–98% identity compared with the other insect sequences and 67% compared to human GCNF), the CTE (100% identity with all insect sequences) and the short F domain (100% identity with coleopteran species and 79% with lepidopteran and dipteran species). Conversely, the LBD showed lower identity, 73% compared with coleopteran species and 56–52% compared with lepidopterans and dipterans, and even lower (20%) when compared with the human sequence.

3.2. Developmental expression and 20E-responsiveness of BgHR4

As a first step towards the characterization of BgHR4 function, we obtained expression patterns of BgHR4 during the embryonic and nymphal development of B. germanica. During embryogenesis, BgHR4 mRNA presented three peaks of expression. Two of them occurred just after 20E pulses, between days 6 and 7 and days 13 and 16, whereas the expression detected at day 2 did not correlate with any detectable ecdysteroid pulse (Fig. 3A).

During nymphal development, the expression pattern of BgHR4 was obtained in the prothoracic gland (the tissue responsible for the synthesis of ecdysteroids), fat body (the main metabolic tissue) and epidermis (which synthesizes the new cuticle). In all tissues, BgHR4 mRNA was present during the decline of the ecdysteroid titer at the end of each instar (Fig. 3B).

The expression patterns of BgHR4 suggested to us that 20E was involved in its regulation. To test this hypothesis, we measured BgHR4 mRNA levels in abdominal tergites, with their epidermis and adhering fat body tissue, from 1–day-old sixth instar female nymphs, incubated in vitro for 1–10 h in the presence of either 20E (5 × 10⁻⁶ M), the protein synthesis inhibitor cycloheximide (Chx) (10⁻⁴ M), or both 20E and Chx. As shown in Fig. 3C, a peak of BgHR4 mRNA appeared 1 h after the addition of 20E. The mRNA then declined to low levels by 10 h. The 20E-dependent induction was greater and sustained during the incubation time when Chx was added to the medium containing 20E. Chx alone did not produce any effect on BgHR4 expression.

Fig. 2. Domain comparison of B. germanica HR4 with other HR4/GCNF nuclear receptors. Letters above BgHR4 indicate functional domains. Numbers within each domain indicate the number of amino acids. The percentages of identity between corresponding domains of BgHR4 and the other orthologs are indicated below each domain.

Sequences and species considered are BgHR4 from B. germanica (this study), TcHR4 from Tribolium castaneum (accession number: XP_974320), TmHR4 from Tenebrio molitor (AJ005685), MsHR4 from Manduca sexta (AF288088), BmGRF from Bombus mori (AF124981), DmHR4 from Drosophila melanogaster (AY971884), AqHR4 from Anopheles gambiae (XP_318161), AaHR4 from Aedes aegypti (AM773447) and HuGCNF from Homo sapiens (AF004291).
of BgHR3 knockdown nymphs at the end of the last instar. Remarkably, whereas the levels of BgEcr-A and BgRXR were not affected by the absence of BgHR3, those of BgHR4 were dramatically reduced (Fig. 4A), clearly indicating that BgHR3 is necessary for BgHR4 activation.

In contrast, we found an inverse correlation between the expression of BgE75C and BgE75A and the activation of BgHR4 (Fig. 1), suggesting a repressive effect of these BgE75 isoforms upon BgHR4 during the rise of circulating ecdysteroids. Again, we tested this by using RNAi to lower the levels of BgE75 isoforms. However, given that there is functional redundancy between BgE75 isoforms (Mané-Padrós et al., 2008), we used a dsRNA designed to the common hinge and LBD regions and hence able to target all BgE75 isoforms simultaneously. Confirming the hypothesis, knocking down BgE75 expression by RNAi resulted in a strong and premature up-regulation of BgHR4 at day 5 of the last instar (Fig. 4B). Interestingly, the reduction of BgE75 levels also resulted in a strong and premature activation of BgHR3, raising the possibility that the repressive effect of BgE75 on BgHR4 was not direct but rather through the repression of BgHR3. We confirmed this relationship by performing RNAi of BgE75 and BgHR3 simultaneously and showing that, under these conditions, the premature activation of BgHR4 was prevented (Fig. 4C).

Collectively, these results indicate that the expression of BgHR4 is regulated by the interplay of two 20E-dependent nuclear receptors, BgHR3 acting as activator and BgE75 repressing the activation of BgHR4 indirectly through the negative regulation of BgHR3 at the onset of the ecdysteroids pulse.

3.4. Disruption of BgHR4 function by RNAi affects nymphal development

To understand the functional relevance of BgHR4 in B. germanica, we first silenced it by RNAi in vivo by injecting 1 μg of dsBgHR4-1 (Fig. 5A) in the abdomen of freshly eclosed last-instar nymphs. As a consequence, mRNA levels of BgHR4 were dramatically reduced 8 days after the injection, both in the prothoracic gland (Fig. 5B) and in the epidermis and fat body (data not shown). Similar reduction was obtained when a second dsBgHR4-2 was used (Supplemental Fig. 1).

Once demonstrated the effectiveness of the dsRNA treatment, we proceeded to the phenotypic analysis of the BgHR4 knockdown nymphs. Whereas all last instar nymphs treated with dsMock (n = 89) molted into adults with the normal developmental timing, those treated with dsBgHR4-1 (n = 73), although showed normal appearance and behavior, did not molt at the end of the stage, stopped moving, and finally died 24–48 h after the time when the dsMock nymphs molted into adults (Fig. 5C and D). Furthermore, the arrested nymphs showed duplicated and superimposed ectodermal-derived structures, such as mandibles, maxilla and laciniae in the head, as well as the entire tracheal system (Fig. 5E–G). Moreover, histological sections of the abdomen of these nymphs showed the newly formed adult endocuticle and exocuticle layers below the nymphal exocuticle, thus indicating that the nymphal endocuticle layer had been digested (Fig. 5H). The analysis of the newly synthesized cuticle, including the size and disposition of the abdominal bristles, indicated that it was adult-like (data not shown). The failure of BgHR4 knockdowns to molt was not due to an ecdysteroid deficiency as no differences in prothoracic gland (Fig. 5B) and in the epidermis and fat body (data not shown). Similar reduction was obtained when a second dsBgHR4-2 was used (Supplemental Fig. 1).

To ascertain whether the requirement of BgHR4 was restricted to the nymph–adult transition or was necessary for each nymph-nymph transition, we injected 1 μg of dsBgHR4-1 into newly

Fig. 3. Expression patterns of BgHR4 mRNA in B. germanica and effect of 20-hydroxyecdysone (20E). (A) Expression pattern of BgHR4 mRNA during embryonic development. Ecdysteroid (20E) levels (upper part) are redrawn from Maestro et al. (2005). Equal amounts of staged embryos were analyzed by RT-PCR/Southern blotting. (B) Expression patterns of BgHR4 mRNAs during the last two nymphal instars. Ecdysteroid (20E) levels (upper part) are redrawn from Cruz et al. (2003). mRNA levels were analyzed in the prothoracic gland (PG), epidermis (EP) and fat body (FB) by RT-PCR/Southern blotting. (C) Effect of 20E on BgHR4 mRNA levels in abdominal tergites with corresponding epidermis and associated fat body from 1-day-old sixth instar female nymphs. Tergites were incubated in vitro in the presence of either 5 × 10⁻⁸ M of 20E; 10⁻⁴ M of cycloheximide (Chx); or both 20E and Chx for the time indicated. Equal amounts of total RNA from the tissues were analyzed by RT-PCR/Southern blotting. In all cases, a BgHR4 specific probe was used for Southern blotting, and BgActin5C levels were used as a reference. The Southern blots shown in A–C are representative of five replicates.

3.3. The interplay between nuclear receptors BgHR3 and BgE75 regulates BgHR4 expression

The expression patterns and the experiments in vitro suggest that the activation of BgHR4 is under a complex regulatory system involving both positive and negative 20E-dependent factors. As the expression of BgHR3 precedes that of BgHR4 (Fig. 1; see Cruz et al., 2007), we wondered whether BgHR3 would have a regulatory role on BgHR4 expression. We tested this possibility with RNAi experiments, by analyzing the expression of BgHR4 in prothoracic glands

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\caption{Expression patterns of BgHR4 mRNA in B. germanica and effect of 20-hydroxyecdysone (20E). (A) Expression pattern of BgHR4 mRNA during embryonic development. Ecdysteroid (20E) levels (upper part) are redrawn from Maestro et al. (2005). Equal amounts of staged embryos were analyzed by RT-PCR/Southern blotting. (B) Expression patterns of BgHR4 mRNAs during the last two nymphal instars. Ecdysteroid (20E) levels (upper part) are redrawn from Cruz et al. (2003). mRNA levels were analyzed in the prothoracic gland (PG), epidermis (EP) and fat body (FB) by RT-PCR/Southern blotting. (C) Effect of 20E on BgHR4 mRNA levels in abdominal tergites with corresponding epidermis and associated fat body from 1-day-old sixth instar female nymphs. Tergites were incubated in vitro in the presence of either 5 × 10⁻⁸ M of 20E; 10⁻⁴ M of cycloheximide (Chx); or both 20E and Chx for the time indicated. Equal amounts of total RNA from the tissues were analyzed by RT-PCR/Southern blotting. In all cases, a BgHR4 specific probe was used for Southern blotting, and BgActin5C levels were used as a reference. The Southern blots shown in A–C are representative of five replicates.

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emerged fifth (penultimate) and fourth instar nymphs. As in the last instar nymphs, 99% of the BgHR4 fifth nymphal instar knockdowns \((n = 35)\) and 98% of fourth nymphal instar knockdowns \((n = 41)\) were unable to complete the molting process and arrested development with duplicated ectodermal structures. The whole data thus indicate that BgHR4 is required for correct molting throughout the postembryonic development of \(B.\) germanica.

3.5. BgHR4 represses the 20E-dependent cascade at the end of the nymphal instar

The expression pattern of BgHR4 and the phenotype of BgHR4 knockdown nymphs indicate that this gene plays a critical role in the 20E-regulated transcription at the end of the instar. As molting inhibition in BgHR4 knockdown nymphs is not due to ecdysteroid deficiency, we wondered whether this nuclear receptor played an essential role in the regulation of the transcriptional response to the hormone. In this context, it is worth noting that the phenotypic effects observed in BgHR4 knockdown nymphs are indistinguishable from those seen in BgHR3 and BgFTZ-F1 knockdowns (Cruz et al., 2007, 2008), thus suggesting functional relationships between these three nuclear receptors. In \(B.\) germanica, the end of each nymphal instar is characterized by the down-regulation of the 20E-dependent nuclear receptors BgE75A, BgE75B and BgE75E, and the strong up-regulation of BgFTZ-F1 (Cruz et al., 2008; Mané-Padrós et al., 2008). To assess whether BgHR4 was responsible for coordinating such transcriptional response, we analyzed the expression of these nuclear receptors in prothoracic glands from dsBgHR4-1- and dsMock-treated last instar nymphs at the end of the instar (Fig. 7A). Whereas the expression of BgHR3, BgECR-A and BgRXR was not affected in BgHR4 knockdown nymphs, the repression of BgE75A, BgE75B and BgE75E was significantly impaired and the activation of BgFTZ-F1 was totally halted. However, since BgHR3 is also necessary for BgFTZ-F1 expression (Cruz et al., 2008) and its levels are affected by the absence of BgHR4, we wondered whether the lack of BgFTZ-F1 expression was a consequence of the high levels of the BgE75 isoforms that, in turn, would impair the activity of BgHR3. To test this possibility, we determined the BgFTZ-F1 mRNA levels on day 6 of the last nymphal instar in dsBgE75-treated nymphs (a treatment that induces the premature expression of BgHR3 and BgHR4), and in nymphs treated simultaneously with dsBgE75 and dsBgHR4-1 (which induces the expression of BgHR3 but not of BgHR4). As Fig. 7B shows, whereas the levels of BgFTZ-F1 were negligible in dsMock-treated nymphs, those in BgE75 knockdown nymphs were very high, as high as in those treated with dsBgE75 and dsBgHR4-1.

Taken together, these results indicate that the effect of BgHR4 on the activation of BgFTZ-F1 is not direct but rather it acts through the repression of BgE75 expression at the end of the instar, thus allowing the remaining BgHR3 to induce BgFTZ-F1 at this precise moment.

4. Discussion

Using RNAi in vivo in \(B.\) germanica, we previously demonstrated that 20E, acting through its heterodimeric receptor BgECR-A/BgRXR, directly induces the activation of a stereotypic cascade of the nuclear hormone receptors BgE75, BgHR3 and BgFTZ-F1, which controls the developmental progression of the cockroach (Cruz et al., 2006, 2007, 2008; Martín et al., 2006; Mané-Padrós et al., 2008, 2010). In this study, we have isolated and characterized BgHR4 in \(B.\) germanica as a new member of this hierarchy.

4.1. Expression patterns and hormonal regulation of BgHR4

Expression studies show that BgHR4 is always present during the decline of the ecdysteroid pulses that occur during the life cycle of \(B.\) germanica. The expression of BgHR4 during embryogenesis suggest that it could be involved in the dorsal closure and the deposition of the first cuticle on days 6–7 and in the deposition of a subsequent cuticle layer on days 13–17 (Fig. 2A). Interestingly, BgHR4 is also detected on day 2, when no ecdysteroid peak is observed. However, we presume that...
there may be an ecdysteroid pulse around this period because we have also detected transient expressions of two 20E-dependent genes, BgE75A (Mané-Padrós et al., 2008) and BgHR3 (Martín et al., unpublished). In comparison with B. germanica, during the embryogenesis of D. melanogaster, the only insect where the embryonic expression of this factor has been reported, DHR4 is detected for a relatively brief temporal window at 10–14 h after egg laying, coinciding with the decline of the single mid-embryonic ecdysteroid pulse (Sullivan and Thummel, 2003). In contrast with the differences in HR4 expression during the embryonic development of B. germanica and D. melanogaster, the timing of BgHR4 expression in the prothoracic gland, epidermis and fat body during nymphal development of B. germanica is very similar to those described during different post-embryonic stages of the holometabolous insects T. molitor (Mouillet et al., 1999), B. mori (Charles et al., 1999), M. sexta (Weller et al., 2001) and D. melanogaster (Sullivan and Thummel, 2003).

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The expression pattern of BgHR4 suggests that 20E is involved in its regulation. Fat body incubations in vitro confirmed that BgHR4 is activated by 20E and that a putative 20E-inducible inhibitor factor, possibly BgHR4 itself, would be involved in the regulation of BgHR4 expression. Again, this type of 20E-responsiveness is similar to that described for HR4 in M. sexta and A. aegypti (Hiruma and Riddiford, 2001; Cruz et al., 2009). Conversely, in D. melanogaster, although DHR4 is a direct target of 20E, the maximal expression of DHR4 requires the synergistic activity of a 20E-induced protein (King-Jones et al., 2005; Gauhar et al., 2009).

4.2. Role of BgHR4 in the ecdysteroid-triggered cascade of B. germanica

In B. germanica, periodic pulses of 20E at the end of each nymphal instar act as developmental timer triggering the molt to the next stage. 20E functions through a complex cascade of nuclear receptors that responds to, and transduces the hormonal signal. The results presented here, clearly demonstrated that BgHR4 contributes decisively to the crossregulatory interactions among these nuclear receptors (Fig. 8).

This complex hierarchy is initiated by the increase of ecdysteroid levels at mid-nymphal instar, which sequentially induces BgE75C, BgE75A and BgE75B as a result of a regulatory crosstalk between them (Mané-Padrós et al., 2008). These BgE75 isoforms, in turn, repress the early activation of BgHR3 until ecdysteroids reach the highest levels (Fig. 4B) (Mané-Padrós et al., 2010). The specific BgE75 isoform responsible for BgHR3 repression has not been identified, but probably all three would have repressor properties given that our previous experiments of BgE75 isoform-specific interference have shown that all isoforms have redundant functions (Mané-Padrós et al., 2008). At the peak of the ecdysteroid pulse, BgE75 isoforms cannot longer inhibit BgHR3 expression. BgHR3 then activates BgHR4 as the ecdysteroid titer declines.
Finally, BgHR4 down-regulates BgE75A, BgE75B and BgE75E (Fig. 7A), allowing BgHR3 to induce BgFTZ-F1 at the end of the nymphal stage (Fig. 8).

Interestingly, the 20E-triggered cascade described above is also present in more derived holometabolous insects, such as M. sexta and D. melanogaster (King-Jones et al., 2005; Hiruma and Riddiford, 2009). However, although the architecture of the hierarchy is mostly conserved, the regulatory interplay between E75, HR3, HR4 and FTZ-F1 differs between hemimetabolous and holometabolous species. Although in D. melanogaster this cascade is recurrently present following the mid-embryonic, second and third-instar ecdysteroids pulses, it has been mainly characterized during the early stages of metamorphosis. Thus, in the prepupal stage of the fruitfly, as happens in B. germanica, DHR3 and DHR4 act together to induce βFTZ-F1 expression (Lam et al., 1999; King-Jones et al., 2005). However, in contrast to the cockroach, DHR4 expression in D. melanogaster begins before that of DHR3, thus suggesting that the DHR4 activation is independent of DHR3 (Sullivan and Thummel, 2003; King-Jones et al., 2005). In M. sexta, GV1 cell transfection assays demonstrated that, as occurs in B. germanica, ME75A represses the induction of MHR3 (Hiruma and Riddiford, 2004). However, two features vary from B. germanica. First, MHR3 represses the 20E-dependent induction of MHR4 (Hiruma and Riddiford, 2007), and second, MHR4 would act as a transcriptional repressor of MβFTZ-F1 (Riddiford et al., 2003). Furthermore, although there is no functional analysis of AaHR3 and AaHR4 in the mosquito A. aegypti, the expression patterns of both receptors suggest that the same regulatory interplay would also occur in the mosquito fat body during adult vitellogenesis (Cruz et al., 2009).

4.3. The function of BgHR4 in molting is channelled through BgFTZ-F1

Using RNAi in vivo, we have shown that BgHR4 is required at the end of each nymphal instar to complete the molting process. BgHR4 knockdown nymphs arrest development with the new adult endo- and exocuticle layers formed and the old nymphal endocuticle totally digested (Fig. 5H). The same molting defects were observed in B. germanica nymphs with reduced levels of BgHR3 and BgFTZ-F1 (Cruz et al., 2007, 2008), which suggest that BgHR3, BgHR4 and BgFTZ-F1 are mainly required for the last step of molting, namely the ecdysis. During this process, a succession of precise body contractions is triggered and controlled by a number of peptides synthesized and released from the CNS and from highly specialized tracheal cells, called Inka cells (Zitnan and Adams, 2005). Interestingly, the molting impairment observed in B. germanica has been also reported in D. melanogaster when βFTZ-F1 levels are reduced in Inka cells by RNAi, as the release of...
ecdysone triggering hormone (ETH) from these cells is blocked (Zitnan et al., 2007). Taken together, these results suggest that in B. germanica, as in D. melanogaster, ecdysin is mainly controlled by BgFTZ-F1 and that the role of BgHR3 and BgHR4 is to provide the necessary delay in BgFTZ-F1 expression until the end of the instar. It is interesting to note, however, that the synthesis of the new cuticle layers and the digestion of the old endocuticle, although 20E-dependent in B. germanica, are two processes that are independent of BgHR3, BgHR4 or BgFTZ-F1, which suggests the occurrence of other 20E-dependent regulators of the molting cycle yet to be identified.

4.4. Conserved and new functions for the HR4 nuclear receptor

The data presented here is the first functional analysis of the HR4 gene in a hemimetabolous insect. In holometabolous species, HR4 loss of function analysis has been carried out in the coleopteran T. castaneum and in the dipteran D. melanogaster. In T. castaneum, TcHR4 is required for normal larval-pupal transition and for successful adult reproduction (Tan and Palli, 2008; Xu et al., 2010). In the more derived D. melanogaster, disruption of DHR4 function during post-embryonic development results in two different phenotypes: first, DHR4 last instar mutant larvae display premature wandering behavior and precocious onset of pupariation; and second, DHR4 mutants arrest development at early stages of metamorphosis mainly due to the absence of a global repression of the ecdysteroid-regulated gene expression (King-Jones et al., 2005). Remarkably, whereas in B. germanica BgHR4 function is necessary to complete all the post-embryonic transitions (this study), DHR4 has no essential functions before the pupal stage in the fruitfly, thereby suggesting that this gene has evolved from an ancestral function related with the control of each developmental transition in hemimetabolous insects to a new holometabolous-specific role in coordinating growth and maturation during the last larval stage. In contrast to this specific new role of DHR4, specifically during the prepupal stage of D. melanogaster DHR4 has maintained its primitive regulatory function observed in B. germanica, acting as a potent transcriptional repressor of several 20E-dependent genes, namely E7SA, E7SB and IMP-L1 among others, and also activating βFTZ-F1 expression (King-Jones et al., 2005). Interestingly, the repressive function of HR4 has been also observed in M. sexta (Hiruma and Riddiford, 2007).

In summary, our work shows that the nuclear receptor BgHR4 is essential throughout nymphal development in the hemimetabolous insect model B. germanica, acting as potent repressor in the 20E-responsive transcriptional cascade that occurs periodically during the post-embryonic development of this cockroach. Furthermore, the comparison of HR4 functions between hemimetabolous and holometabolous species shows that this nuclear receptor may have played a major role in the selection of new-specific regulatory processes associated to the evolution towards holometabolob.

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Appendix A. Supplementary data


References


