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Redundant ecdysis regulatory functions of three nuclear receptor HR3 isoforms in the direct-developing insect *Blattella germanica*

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Abstract

In hemimetabolous insects, the molecular basis of the 20-hydroxyecdysone (20E)-triggered genetic hierarchy is practically unknown. In the cockroach *Blattella germanica*, we had previously characterized one isoform of the ecdysone receptor, *BgEcR-A*, and two isoforms of its heterodimeric partner, *BgRXR-S* and *BgRXR-L*. One of the early-late genes of the 20E-triggered genetic hierarchy, is *HR3*. In the present paper, we report the discovery of three isoforms of HR3 in *B. germanica*, that were named BgHR3-A, BgHR3-B_1 and BgHR3-B_2. Expression studies in prothoracic gland, epidermis and fat body indicate that the expression of the three isoforms coincides with the peak of circulating ecdysteroids at each nymphal instar. Experiments *in vitro* with fat body tissue have shown that 20E induces the expression of BgHR3 isoforms, and that incubation with 20E and the protein inhibitor cycloheximide does not inhibit the induction, which indicates that the effect of 20E on *BgHR3* activation is direct. This has been further confirmed by RNAi *in vivo* of BgEcR-A, which has shown that this nuclear receptor is required to fully activate the expression of BgHR3. RNAi has been also used to demonstrate the functions of BgHR3 in ecdysis. Nymphs with silenced *BgHR3* completed the apolysis but were unable to ecdyse (they had duplicated and superimposed the mouth parts, the hypopharinge, the tracheal system and the cuticle layers). This indicates that BgHR3 is directly involved in ecdysis. Finally, RNAi of specific isoforms has showed that they are functionally redundant, at least regarding the ecdysis process.

Keywords: HR3; Nuclear hormone receptor; *Blattella germanica*; 20-Hydroxyecdysone; RNAi; Ecdysis

1. Introduction

In insects, the ecdysteroidal hormone 20-hydroxyecdysone (20E) controls key developmental processes during embryogenesis, molting, metamorphosis and reproduction. Among these processes, metamorphosis in holometabolous species, particularly in the fruitfly *Drosophila melanogaster*, has been extensively studied. At the molecular level, 20E exerts its regulatory functions upon binding to a heterodimeric receptor formed by two members of the nuclear receptor superfamily, the ecdysone receptor (EcR) and the retinoid-X-receptor (RXR) ortholog ultraspiracle (USP) (Yao et al., 1993). Once activated, the receptor elicits cascades of gene expression that mediate and amplify the ecdysteroidal signal. Most of the early response genes encode transcription factors, like E75, E74 and Broad, which in turn regulate later genes (reviewed by Thummel, 1995; Riddiford et al., 2001; King-Jones and Thummel, 2005). Remarkably, the activity of the early genes at the onset of the pupal stage is further refined, in terms of timing and specificity of activation, by the presence of other 20E-dependent genes, namely *HR3* (or *Hr46*) and βFTZ-F1 (Lam et al., 1997, 1999; Broadus et al., 1999).

The situation is very different in hemimetabolous insects, which do not develop through complete metamorphosis and whose juvenile forms are morphologically similar to the adult, only differing, in general, by color, wing and genitalia details. Indeed, with the exception of few and isolated studies (Erezyilmaz et al., 2006), data concerning the molecular basis of the 20E-triggered genetic

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hierarchy is practically non-existent in hemimetabolous insects. However, if we aim to understand the molecular basis of the evolution towards complete metamorphosis in insects, then characterization of the 20E-induced genetic hierarchy in hemimetabolous species becomes of paramount importance.

In this context, we started isolating the components of the 20E receptor in the hemimetabolous cockroach *Blattella germanica*. Firstly, we cloned one isoform of the ecdysone receptor, *BgEcR-A*, and two isoforms of its heterodimeric partner, *BgRXR-S* and *BgRXR-L*, and we found that they are expressed following a housekeeping-like pattern during the last two nymphal instars (Cruz et al., 2006; Maestro et al., 2005). Furthermore, silencing of BgEcR-A and both isoforms of BgRXR by RNA interference (RNAi) *in vivo*, showed that these nuclear receptors are essential for nymphal development (Cruz et al., 2006; Martín et al., 2006).

One of the genes following the 20E-triggered genetic hierarchy, and which has been functionally characterized in *D. melanogaster*, is *HR3*, again a member of the nuclear receptor superfamily. The corresponding mammalian ortholog of *HR3* is the RAR-related orphan receptor (ROR) (Giguere et al., 1994). In *D. melanogaster*, *HR3* resets the genetic cascade triggered by 20E at level of the early genes, repressing their expression at the prepupal stage, while activating the nuclear receptor βFTZ-F1, which provides competence to respond to 20E in late prepupae (Woodard et al., 1994; Lam et al., 1997; White et al., 1997; Kageyama et al., 1997; Broadus et al., 1999).

Phenotypically, mutation analysis of *HR3* in *D. melanogaster* has revealed that it is required for metamorphosis (Carney et al., 1997), in particular for the correct prepupal–pupal transition and for the differentiation of adult structures (Lam et al., 1999). Orthologs of *D. melanogaster HR3* have been reported in other holometabolous insects, like in the dipteran *Aedes aegypti* (Kapitskaya et al., 2000), and the lepidopterans *Manduca sexta* (Palli et al., 1992), *Galleria mellonella* (Jindra et al., 1994), *Bombyx mori* (Eystathioy et al., 2001), *Choristoneura fumiferana* (Palli et al., 1996, 1997) and *Helicoverpa armigera* (Zhao et al., 2004).

In the present work, we extend the knowledge of the 20E genetic hierarchy in the hemimetabolous *B. germanica* by identifying three HR3 homologs in this cockroach, by studying their regulation by ecdysteroids and by determining their functions in nymphal development using RNAi approaches.

2. Results

2.1. Cloning of HR3 isoforms in Blattella germanica

Cloning of BgHR3 cDNAs 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 sequences of insect HR3 orthologs. Using cDNA from *B. germanica* UM-BGE-1 cells as a template, a 129 bp PCR fragment was obtained, and its sequence was highly similar to insect HR3 sequences. 3′-RACE and 5′-RACE experiments using cDNA from UM-BGE-1 cells, gave three full-length cDNAs of 3.50, 3.79 and 2.36 kb. Database BLAST search with the complete sequences revealed that they encoded *B. germanica* orthologs of HR3. Two HR3 cDNAs encoded identical proteins except for an insertion/deletion of 121 amino acids in the D domain (hinge region), and were named BgHR3-B_1 (accession number: AM259129) and BgHR3-B_2 (accession number: AM259130). The third BgHR3 differed from the other two sequences in the 5′UTR and in most of the A/B domain, and was named BgHR3-A (accession number: AM259128). With the exceptions mentioned, the three BgHR3 are identical not only in terms of amino acids, but also at level of nucleotide sequence, which suggests that they are splice variants of the same gene.

BgHR3-A encodes a 607 amino acid protein with a predicted molecular mass of 68 kDa, whereas *BgHR3-*B_1 and *BgHR3-*B_2 encode two proteins of 651 and 530 amino acids with predicted molecular masses of 72.9 and 59.2 kDa, respectively. In all sequences, putative start codons are preceded by in-frame stop codons, indicating that these sequences represented full-length open reading frames (ORFs). To verify that the cloned cDNAs contained translatable ORFs, they were expressed in a coupled TNT system under the control of the SP6 promoter. SDS–PAGE and fluorography showed that the molecular sizes of the proteins synthesized *in vitro* closely corresponded to those expected.

In general, nuclear hormone receptor isoforms are based on divergences in their A/B domains. Therefore, we carried out a maximum-likelihood analysis of the A/B domain for all insect HR3 sequences reported to date, except DHR3B from *D. melanogaster*, which has a very short A/B domain. The analysis generated the tree shown in Fig. 1a, which clusters the sequences into three main groups, A, B and C, with notably high bootstrap support values (782 for nodes A and C, and 744 for node B, with respect to 1000 replicates). Classification of *B. germanica* HR3 isoforms into A and B categories was based on that analysis.

Aside from the A/B domain, amino acid sequence comparisons revealed that the three cDNAs include the domain structure characteristic of the members of the nuclear hormone receptor superfamily. The comparison with other HR3/ROR sequences (Fig. 1b), revealed that the most conserved domains are the DBD (92–96% similarity compared to other invertebrates and 77% compared to human RORα), and a 23 amino acids region named carboxy-terminal extension (CTE) of the DBD (91–100% and 73% similarity, respectively). Conversely, the ligand binding domain (LBD) of BgHR3 showed lower similarity with other insect orthologs, 60% compared with dipteran species and 55% with lepidopterans. Similarity was still lower when compared with nematode (27%) and human (35%) sequences. However, the LBD contains the highly