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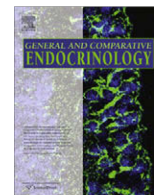

Interactions of the crustacean nuclear receptors HR3 and E75 in the regulation of gene transcription

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ABSTRACT

Endocrine signal transduction occurs through cascades that involve the action of both ligand-dependent and ligand-independent nuclear receptors. In insects, two such nuclear receptors are HR3 and E75 that interact to transduce signals initiated by ecdysteroids. We have cloned these nuclear receptors from the crustacean *Daphnia pulex* to assess their function as regulators of gene transcription in this ecologically and economically important group of organisms. Both nuclear receptors from *D. pulex* (DappuHR3 (group NR1F) and DappuE75 (group NR1D)) exhibit a high degree of sequence similarity to other NR1F and NR1D group members that is indicative of monomeric binding to the RORE (retinoid orphan receptor element). DappuE75 possesses key amino acid residues required for heme binding to the ligand-binding domain. Next, we developed a gene transcription reporter assay containing a luciferase reporter gene driven by the RORE. DappuHR3, but not DappuE75, activated transcription of the luciferase gene in this system. Co-transfection experiments revealed that DappuE75 suppressed DappuHR3-dependent luciferase transcription in a dose-dependent manner. Electrophoretic mobility shift assays confirmed that DappuHR3 bound to the RORE. However, we found no evidence that DappuE75 similarly bound to the response element. These experiments further demonstrated that DappuE75 prevented DappuHR3 from binding to the response element. In conclusion, DappuHR3 functions as a transcriptional activator of genes regulated by the RORE and DappuE75 is a negative regulator of this activity. DappuE75 does not suppress the action of DappuHR3 by occupying the response element but presumably interacts directly with the DappuHR3 protein. Taken together with the previous demonstration that daphnid HR3 is highly induced by 20-hydroxyecdysone, these results support the premise that HR3 is a major component of ecdysteroid signaling in some crustaceans and is under the negative regulatory control of E75.

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1. Introduction

The ecdysteroid signaling pathway is a major component of the endocrine axis controlling development and reproduction in arthropods. Numerous nuclear receptors, transcription factors, coactivators, and corepressors coordinate within this pathway to transmit the ecdysteroid-induced signal. Much is known about the structure, function, and interplay of these signaling components in insects (Smagghe, 2009). Much less is understood about the genomic signaling pathway of ecdysteroid signaling in crustaceans, despite the significant ecological and economic importance of this subphylum and the demonstrated ability of many environmental contaminants to interfere with this pathway in these organisms (LeBlanc, 2007; Tuberty and McKenney, 2005; Zou, 2005). A definitive understanding of ecdysteroid signaling pathways in crustacean is integral to protecting these organisms against endocrine disruptive environmental factors.

Ecdysteroids bind and activate the nuclear receptor heterodimer consisting of the ecdysteroid receptor (EcR) and the retinoid X receptor or ultraspiricle (RXR/USP) (Koelle et al., 1991; Riddiford et al., 2000; Yao et al., 1992). This ligand-bound heterodimer activates transcription of a cascade of genes that regulate many physiological events including metamorphosis (Bialecki et al., 2002; Thummel, 1996), embryogenesis (Bownes et al., 1988), growth (Bownes et al., 1988), differentiation (Laufer et al., 2002), egg chamber development (Buszczak et al., 1999), ecdysis (Ampleford, 1985), diapause, reproduction, and behavior (Richard et al., 1998). Two important transcriptional regulators in insect ecdysteroid signaling cascades are the nuclear receptors HR3 (group NR1F)

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and E75 (group NR1D) (King-Jones and Thummel, 2005). HR3 activates target downstream genes in the signaling pathway and E75 is best recognized as a negative regulator of HR3 transcriptional activation (Hiruma and Riddiford, 2004; Swevers et al., 2002; White et al., 1997). In *Drosophila melanogaster*, the interaction between HR3 and E75 is further regulated by nitric oxide or carbon monoxide, which binds to the heme moiety associated with E75 (Reinking et al., 2005). It is currently unknown whether a similar interplay of receptors operates in the ecdysteroid signaling pathway of crustaceans.

Studies involving receptor signaling in crustaceans of the genus *Daphnia* have been greatly facilitated by the recent sequencing of the *Daphnia pulex* genome (http://wFleaBase.org). We identified 25 nuclear receptor genes in the *D. pulex* genome (Thomson et al., 2009). Many of these receptors are orthologs to insect receptors involved in the ecdysteroid signaling pathway. In particular, we identified sequences for both E75 and HR3. We cloned, sequenced, and characterized expression patterns of these nuclear receptors from the closely related species, *Daphnia magna* (Hannas and LeBlanc, 2009). The receptor sequences indicate that the proteins may contain structural characteristics similar to those of the *Drosophila* orthologs, suggesting that they play similar roles to the *Drosophila* receptors in ecdysteroid signaling.

The goal of the present study was to functionally characterize these receptors by determining: (1) if either HR3 or E75 cloned from *D. pulex* (DappuHR3 and DappuE75, respectively) activate transcription of a reporter gene under the control of the RORE and (2) if any regulatory interactions occur between DappuHR3 and DappuE75.

2. Materials and methods

2.1. Full-length E75 and HR3 derivation

Female daphnids ((*D. pulex*) (clone MP2 (Busey 16)) provided by Dr. Jeffery Dudycha, University of South Carolina, USA), were cultured as described previously (Rider et al., 2005). Adults (>2 weeks after birth) were stored in RNAlater® (Ambion, Austin, TX, USA) at −20 °C until sufficient tissue mass was collected for RNA isolation (approximately 30 mg wet weight). Daphnids were homogenized and RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI). RNA integrity was confirmed by formaldehyde agarose gel electrophoresis. The concentration of RNA was determined by absorbance at 260 nm and the purity determined by the 260/280 nm absorbance ratio, using a Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Montchanin, DE). The ImProm-II™ Reverse Transcription System (Promega) and oligo dT primers were used to reverse transcribe RNA into cDNA.

Primers were designed at the 5′ and 3′ ends of the predicted open reading frame (ORF) for both E75 and HR3 genes derived from the *D. pulex* genome (Thomson et al., 2009). The primer sequences used were as follows:

DappuE75 F: 5′-**GACGACGACAAG**ATGAGAAGTGAAATTGTTGTG-3′,
DappuE75 R: 5′-**GAGGAGAAGCCCGGT**TCAGCCCTTCATGATGTTGG-3′,
DappuHR3 F: 5′-**GACGACGACAAGATG**ATGGAAGCTCCGGCCGTT-3′,
DappuHR3 R: 5′-**GAGGAGAAGCCCGGT**TCAACTATCCACGGAAAAGAG-3′.

The bold portion of each primer corresponds to a ligase-independent cloning (LIC) extension sequence for cloning into an Ek/LIC vector (Novagen, EMD Biosciences, San Diego, CA, USA) which can be used for recombinant protein expression in *Escherichia coli*. The genes were amplified by PCR using 75 ng cDNA, 22.5 μL high fidelity Supermix (Invitrogen, Carlsbad, CA), and 0.4 μM primers in a final volume of 25 μL. Cycling conditions were as follows: denature for 30 s at 94 °C, anneal primers for 30 s at 57 °C, and extend products for 3 min at 72 °C for a total of 40 cycles, followed by a final extension for 7 min at 72 °C. PCR products were purified from a 1.2% agarose gel using the Wizard® SV Gel and PCR Clean-up System (Promega). Purified products were cloned into the vector pCR®4-TOPO using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. The gene inserts were subsequently sequenced by primer extension (SeqWright Inc., Houston, TX). The amino acid sequence and molecular weight for both the DappuHR3 and DappuE75 proteins were determined using ExPASy software (http://www.expasy.org/). The DappuHR3 sequence was aligned with the ortholog from *D. magna* and the *D. pulex* HR3 sequence predicted from the genome annotation using ClustalW2 software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The DappuE75 protein sequence was aligned with that of *D. magna*, the tropical landcrab *Gecarcinus lateralis*, and the sand shrimp *Metapenapeus ensis* (Accession numbers are available in Additional File 1). Prediction of the DNA-binding domain and ligand-binding domain locations within the sequence were made based on the DappuE75 and DappuHR3 sequences, using NCBI protein BLAST (Altschul et al., 1997).

Following release of an update of the *D. pulex* genome, we discovered that the above cloning of DappuE75 yielded a gene sequence that was missing 403 nucleotides in the C-terminus and this cDNA was used to produce proteins for the functional characterization of the receptors. Therefore, the primers E75 R2 (full length): 5′-**GAGGAGAAGCCCGGT**TCAGGCGTGAAGGGGAAAAT-3′ and E75 F from above were used in PCR (as above) to obtain the full-length cDNA which was used in comparative functional experiments with the truncated cDNA.

2.2. Phylogenetic analysis

Phylogenetic analyses of DappuHR3 and DappuE75 receptors were performed using methods described previously (Thomson et al., 2009) with some modification. First, the DNA-binding domain (DBD) and the ligand-binding domain (LBD) of each receptor used in the analyses were identified using the conserved domain database (CDD) (Marchler-Bauer et al., 2007); DBD and LBD were joined; and, aligned using the default parameters of ClustalX (Thompson et al., 1997). Alignments and phylogenetic analysis of only the DBD or LBD also were performed and are presented in Additional Files 4–7. Receptor sequences from *D. pulex* were compared to those of other species available in GenBank (NCBI accession numbers for the receptors used are available in Additional File 1).

Trees were constructed using Bayesian Inference with MrBayes software version 3.1.2 (Ronquist and Huelsenbeck, 2003) using two computing clusters: Bioportal (www.bioportal.uio.no) run by the University of Oslo; and, the Computational Biology Service Unit of Cornell University (http://cbsuapps.tc.cornell.edu/mrbayes.aspx). Phylogenetic trees were constructed using the "mixed-model" approach in which the Markov chain Monte Carlo sampler explores nine different fixed-rate amino acid substitution models implemented in MrBayes. We used four chains with runs of 5 million generations, chains sampled every 100 generations, a burnin of 10,000 trees with the *WAG* model (Whelan and Goldman, 2001). Phylogenies were rooted to the *Caenorhabditis elegans* receptors as the most ancient nuclear receptor on the tree.

Maximum parsimony and neighbor-joining (NJ) distance parameters were used to provide additional phylogenetic support for phylogenetic relationships observed among the nuclear receptors. Unrooted parsimony was constructed using PAUP version

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