Molecular cloning and sequence of retinoid X receptor in the green crab Carcinus maenas: a possible role in female reproduction

Ganji Purna Chandra Nagaraju*, Balney Rajitha* and David W Borst

Department of Biology, University of Central Florida, Orlando, Florida 32816, USA

(For correspondence should be addressed to G P Nagaraju; Email: pganji@emory.edu)

G P Nagaraju is now at Department of Hematology and Medical Oncology, Emory University, Atlanta, Georgia 30322, USA.

*G P Nagaraju and B Rajitha contributed equally to this work.

Abstract

Retinoid X receptor (RXR) belongs to an ancient super-family of nuclear hormone receptors, and plays an important role in reproduction of vertebrates. However, the reproductive role of RXR has not been clarified in crustaceans. In this investigation, we first report the cloning of two alternative splice variants of RXR cDNA from green crab ovarian RNA. RXR mRNA levels were quantified in different vitellogenic stages of the crab hepatopancreas (HP) and ovary. The expression of RXR mRNA relative to the arginine kinase mRNA was significantly increased in the HP of vitellogenic crabs in a stage-dependent manner. The relative levels of RXR mRNA in the ovary were significantly lower in vitellogenic stage III crabs than in crabs in the other three stages. These data indicate that the HP and ovary of the crab are capable of expressing RXR, which may regulate, in part, vitellogenesis in the crab. We also examined the effects of methyl farnesoate (MF) and RXR-dsRNA treatments on vitellogenin and RXR gene expression. Vitellogenin and RXR mRNA levels in HP and ovarian fragments incubated in MF were significantly \( (P<0.001) \) higher than in control tissue fragments prepared from the same animal. Treatment of crabs with RXR-dsRNA significantly \( (P<0.001) \) reduced mRNA levels for RXR and for vitellogenin as well as MF levels in hemolymph. These results indicate that, MF and RXR form a complex (MF–RXR) directly and together stimulate ovarian development in these green crabs. This interaction of RXR, MF, and ovary development axis is a novel finding and is the first report to the best of our knowledge.

Journal of Endocrinology (2011) 210, 379–390

Introduction

Retinoid X receptor (RXR) belongs to an ancient super-family of nuclear receptor proteins \((\text{Oro et al. } 1988)\). In vertebrates, three RXR proteins have been reported: \(\text{RXR}_\alpha, \text{RXR}_\beta, \text{and RXR}_\gamma\) and are encoded by three genes: \(\text{RXR}_\alpha, \text{RXR}_\beta, \text{and RXR}_\gamma\). RXR proteins contain up to six domains depending on the species and tissue source. Of the six domains (A–F), the C domain (DNA-binding domain) that contains two C2C2 zinc-finger motifs \((\text{Aranda \\& Pascual } 2001, \text{Devarakonda et al. } 2003)\) and the E domain (ligand-binding domain (LBD)) are present in all nuclear receptors. RXR regulates several physiological and metabolic functions in vertebrates, including growth, differentiation, and reproduction \((\text{Evans } 1988, \text{Mark \\& Chambon } 2003, \text{Mark et al. } 2006)\).

RXR proteins are also found in invertebrates, including insects, crustaceans, and mollusks \((\text{Chung et al. } 1998a, \text{Hayward et al. } 1999, \text{Nishikawa et al. } 2004, \text{Bouton et al. } 2005, \text{Castro et al. } 2007)\). In crustaceans, RXR proteins have 402–442 amino acid residues and show a considerable degree of sequence similarity to each other and to vertebrate RXR proteins \((\text{Kim et al. } 2005)\). Of particular interest is the conservation of C and D domains at the same relative positions in vertebrates and invertebrates \((\text{Kim et al. } 2005)\). Although it has been proposed that RXR isoforms may regulate different physiological functions \((\text{Hopkins et al. } 2008)\), the significance of such a structural polymorphism is far from clear.

The crustacean sesquiterpenoid methyl farnesoate (MF) is a unepoxidated form of juvenile hormone (JH) III. It is produced by the mandibular organ (MO) of more than 35 crustacean species. It is a major signaling molecule in crustaceans with wide-ranging roles in regulating aspects of their physiology \((\text{Nagaraju } 2007)\). Nevertheless, the characteristics of a MF receptor remain uncertain. In some crustaceans RXR binds to sesquiterpenes, for example, in Uca pugilator (U. pugilator) both 9-eis retinoic acid (RA) and MF bind with high affinity to RXR \((\text{Hopkins et al. } 2008)\). The ability of various terpenoids at low concentrations to bind invertebrate RXRs suggests that like the vertebrate RXRs, the invertebrate RXRs are responsive to multiple sesquiterpenes \((\text{Kostrouch et al. } 1998, \text{Wang \\& LeBlanc } 2009)\). Furthermore, Wang & LeBlanc \((2009)\) demonstrated...
that the daphnia RXR is subject to ligand-dependent activation (as demonstrated with tributyltin) and MF does not activate RXR-mediated gene transcription. However, MF synergizes with ecdysteroids to enhance gene transcription mediated by the RXR–ecdysteroid receptor (EcR) complex (Hopkins et al. 2008). This result is well-matched with the recent observation by Wang & LeBlanc (2009) that sequesterpenoids synergize with 20-hydroxyecdysone to increase the activation of the RXR–EcR heterodimeric transcription factor. So MF has been proposed to be a natural ligand for a crustacean RXR (LeBlanc 2007, Wang et al. 2007).

The green crab, Carcinus maenas (C. maenas), has been labeled one of the 100 worst invasive species (Lowe et al. 2000). This euryhaline crab lives in saltwater with salinities from 4 to 52 parts per trillion (ppt) and in temperatures from 0 °C to 30 °C (Cohen & Carlton 1995). In a previous study, we showed that environmental factors differentially affect MF levels in the two color phases of green crab. Furthermore, these differences may be important in stimulating testicular development in one of the color phases (Nagaraju & Borst 2008). Likewise, a better understanding of female reproductive processes and their molecular regulation is of potential importance. Such information would increase our basic understanding of crustacean reproduction and may provide new strategies for controlling crustacean populations.

So far, there are only partial nucleotide sequences of the green crab RXR cDNAs (GenBank accession numbers AY496928, DN739137, and DN739136; Chung & Webster, unpublished data; Towle & Smith 2006) available in Genbank. In this study, we report the cloning of two splice variants of RXR cDNA from green crab ovarian RNA. We used these data to design a quantitative real-time PCR (qPCR) method to measure RXR mRNA levels in green crab ovary and hepatopancreas (HP) at different stages of vitellogenesis. Finally, we show that silencing RXR mRNA with dsRNA disrupts the vitellogenic cycle, indicating that RXR plays an essential role in the green crab vitellogenesis.

Materials and Methods

Animals

Female adult green crabs, C. maenas (Linnaeus 1758), are generously provided by Dan Landers (Millstone Environmental Lab, Waterford, CT, USA). The green crabs were maintained in large tanks containing artificial seawater (Instant Ocean; Spectrum Brands, Atlanta, GA, USA) at normal salinity (33 ppt) at 18 °C in the dark. Crabs were fed on shrimp twice weekly and were acclimated to the tanks for at least a week before being used in experiments.

Tissue collection for RNA isolation

The crab body wet weights were measured and then tissues and organs were removed and transferred to ice-cold crustacean physiological saline (975 mOsm/kg; Pantin 1934). For one study, we collected and analyzed a variety of tissues (see below). However, in most studies, we collected only the HP and the ovaries (OV). For the ovary samples, adhering tissue was removed and the wet weight of each crab ovary was measured. The value was used to calculate the ovarian index (OI; percentage of ovarian weight of the total body weight) for that crab. Samples of each tissue (200 mg) were placed in RNAlater (Ambion) and held at −80 °C until processed.

Total RNA isolation and first-strand cDNA synthesis

Tissues were homogenized in TRIzol (Life Technologies, Inc.), and total RNA was isolated according to the manufacturer’s instructions. The reverse transcription (RT) step was performed with MultiScribe reverse transcriptase (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s instructions.

Cloning and sequencing of RXR cDNA

We used the three partial nucleotide sequences of the green crab RXR cDNAs (GenBank accession numbers AY496928, DN739137, and DN739136) to design a pair of primers (CmRXR_F1 and CmRXR_R1; Table 1). The primers (0.2 μM) were used in standard PCR reactions (25 μl total volume) with an initial denaturation (94 °C, 3 min), and 33 amplification cycles of annealing (58 °C, 30 s), extension (72 °C, 30 s), and denaturation (94 °C, 30 s). The last cycle included a 5 min extension.

Table 1 Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Length</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RXR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CmRXR_F1</td>
<td>24</td>
<td>TACGGCGTGTACAGTTGTGGAGGA</td>
</tr>
<tr>
<td>CmRXR_R1</td>
<td>24</td>
<td>GCCAAAACACATCCCCACACTCCA</td>
</tr>
<tr>
<td>CmRXR_F2</td>
<td>20</td>
<td>CCATGGGGATGAAGAGGAA</td>
</tr>
<tr>
<td>CmRXR_R2</td>
<td>24</td>
<td>TGTGATCGACGTCCGTCGAGATA</td>
</tr>
<tr>
<td>CmRXR_F3</td>
<td>24</td>
<td>ACAGTTGCCAAGGACCTGACTTAT</td>
</tr>
<tr>
<td>CmRXR_R3</td>
<td>24</td>
<td>TTCTGTTGCCAGAACATCTGGCA</td>
</tr>
<tr>
<td>CmRXR_F4 (UTR)</td>
<td>22</td>
<td>GGATTCTCAACGAGACTTCCTC</td>
</tr>
<tr>
<td>CmRXR_R4 (UTR)</td>
<td>22</td>
<td>CGAGCCTTCTGCTTTATCTTCC</td>
</tr>
<tr>
<td>GFP_F1</td>
<td>20</td>
<td>ATGGTGAGCAAGGGCGAGGA</td>
</tr>
<tr>
<td>GFP_R1</td>
<td>20</td>
<td>TTACCTTGACATCCGCTTC</td>
</tr>
<tr>
<td>CmRXR dsRNA_F</td>
<td>42</td>
<td>TAATACGACTCACTATAGGGT</td>
</tr>
<tr>
<td>CmRXR dsRNA_R</td>
<td>42</td>
<td>CTGCGAGAGCTTCCTTC</td>
</tr>
<tr>
<td>GFP dsRNA_F</td>
<td>42</td>
<td>AGAAGAAGGATTTCCAGAC</td>
</tr>
<tr>
<td>GFP dsRNA_R</td>
<td>42</td>
<td>TTACGACTCACTATAGGGT</td>
</tr>
<tr>
<td>Vitellogenin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CmVg_F1</td>
<td>20</td>
<td>TGGGGGATCGAACATACAGAAGA</td>
</tr>
<tr>
<td>CmVg_R1</td>
<td>20</td>
<td>ACAGACGCTTGTCCTTC</td>
</tr>
<tr>
<td>Arginine kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CmAK_F1</td>
<td>21</td>
<td>TCACAGAACACAAATGGGCCGAC</td>
</tr>
<tr>
<td>CmAK_R1</td>
<td>24</td>
<td>AGCAGACTGACAAGCACCCCTAA</td>
</tr>
</tbody>
</table>

Journal of Endocrinology (2011) 210, 379–390

www.endocrinology-journals.org
had a longer extension period (72 °C for 8 min), followed by cooling at 4 °C.

For rapid amplification of cDNA ends (RACE) reactions, we reverse transcribed 1 μg of ovarian RNA (from vitellogenic stage I ovary) using reagents and a protocol provided by the Smart RACE cDNA Amplification Kit (Clontech, BD Biosciences, Inc.). For the 3’ RACE, gene-specific primer (GSP; CmRXR_F2; Table 1), and a universal primer mix (UPM) supplied by the RACE Kit was used. For the 5’ RACE, another GSP (CmRXR_R2) and the UPM were used. Both procedures used the SMART RACE cDNA amplification kit protocol.

After PCR amplification, an aliquot of the reaction was separated on a 1-2% agarose gel and the amplified band visualized with ethidium bromide (Biotech grade; Fisher Scientific, Pittsburgh, PA, USA). PCR products were excised from gels, purified with the MinElute PCR Purification Kit (Qiagen) and cloned into M13 (One Shot TOP10; Invitrogen). Recombinant plasmids were extracted (Mini-M Plasmid DNA Extraction System, Qiagen) and sequenced (Elim Biopharma, Hayward, CA, USA). Analysis of nucleotide and amino acid sequences was performed by software provided by BioEdit (http://www.mbio.ncsu.edu/BioEdit/), NCBI database (http://www.ncbi.nlm.nih.gov/), and SDSC Biology work bench 3.2. (http://workbench.sdsc.edu). The full-length cDNA sequences of Carma_RXR1 and Carma_RXR2 were confirmed by PCR using primers based on the sequences of the 5’ and 3’ UTR regions (see Table 1).

Synthesis of dsRNAs

To produce dsRNA targeting Carma_RXR, a 481 bp (437 bp + 44 additional base pairs in accordance with the Ambion protocol) DNA was cloned into the pCR 4-TOPO Cloning Vector (Invitrogen). This fragment encoded the protein sequence specific to the LBD amino acids 266–412, which is common to both Carma RXR1 and RXR2 (see Fig. 1). Thus, treatment with the dsRNA should knockdown both transcripts. The dsRNA was generated by in vitro transcription using CmRXR-dsRNA-F and CmRXR-dsRNA-R (see Table 1) and the T7 Megascript RNAi Kit (Ambion), which were used according to the manufacturer’s recommendations. Both strands were synthesized in the same tube by incubating at 37 °C for 18 h. The tube was then heated to 75 °C for 5 min and cooled down slowly to room temperature to allow strands to hybridize. For a negative dsRNA control, DNA template amplification was performed on the green fluorescent protein (GFP). Formation of dsRNAs was confirmed by running 1 μg of the reaction in a 1-2% agarose gel. After DNase digestion to remove the template and any ssRNA that did not anneal, the dsRNA was purified with a filter cartridge as described in the kit. The dsRNA was eluted with 100 μl of 95 °C Tris buffer (10 mM, pH 7) with 1 mM EDTA, and then brought to room temperature to allow the strands to hybridize. After quantification, the purified dsRNA was aliquoted and stored at −20 °C.

Animal tissue culture (in vitro effect of MF and RXR-dsRNA on RXR and Vg mRNA levels of ovarian and HP tissue)

We modified M199 by adding 9-6 g of M199 powder with l-glutamine and Earle’s salts to 100 ml of dH2O, 2.5 mg of streptomycin, and 2-2 g of NaHCO3. The volume was increased to 1000 ml with Pantin (1934) crustacean saline, modified to compensate for the salts in the culture medium. The modified M199 contained 340 mM NaCl, 12 mM CaCl2, 11 mM KCl, 26 mM NaHCO3, 16 mM MgSO4, and 0.8 mM NaHPO4 and its osmolarity was 975 mOsm/kg. After adjusting the pH to 7–4, the medium was stored at 4 °C until used.

The reproductive stages were identified according to Nagaraju et al. (2003). Briefly, in C. maenas, the previtellogenic ovary is white in color. During vitellogenesis, the color of the ovary changes from pale yellow (vitellogenic stage I) to orange (vitellogenic stage II) and then it becomes brown (vitellogenic stage III) before spawning. Ovary and HP slices (~25 mg) were collected from intermolt (C4 stage), and vitellogenic stage I female green crabs and placed in 1 ml of modified M199 in individual six wells of a tissue culture plate. The slices were divided into ten groups. Groups with odd numbers were ovary slices and even numbers were HP slices. Groups 1 and 2 served as controls, and received no treatment. Groups 3 and 4 were received (by injection) 10 μl of crab physiological saline (Pantin 1934) with GFP. Slices in groups 5 and 6 received MF at a dose of 1 μg/slice in 10 μl volume of 5% ethanol. Slices in groups 7 and 8 received dsRNA (RXR) at a dose of 1 μg/slice in 10 μl volume of crab physiological saline. Slices in groups 9 and 10 received both MF (1 μg/slice) plus dsRNA of RXR (1 μg/slice). Each group was incubated at 24 °C on an orbital shaker (50–60 g) for 6 h. Total RNA was extracted from each slice and used to measure the levels of RXR mRNA, Vg mRNA, and arginine kinase (AK) mRNA.

In vivo effects of MF and RXR-dsRNA on RXR mRNA levels in female green crab

Twenty-five crabs (intermolt, C4 stage (Nagaraju et al. 2004)) were divided into groups of five animals. Group 1 (controls) received no treatment and were killed on the first day that the experiment began. The remaining animals were treated on day 1, 5, 10, and 15 of the experiment. They were all treated by injection through the arthrodial membrane of the coxa of the third walking leg. Group 2 crabs were concurrent controls and received 100 μl of crab physiological saline containing 5% ethanol with GFP. Group 3 animals were treated with MF (1 μg/crab added to 100 μl crustacean physiological saline in 5% ethanol). Group 4 crabs were treated with RXR-dsRNA. The dsRNA was suspended in diethylpyrocarbonate-treated water and diluted with crab physiological saline to a final concentration of 5 μg/100 μl. Group 5 animals were treated with both MF plus RXR-dsRNA. None of the animals died from the treatment. On day 20, the crabs were weighed and
The nucleotide and amino acid full-length sequence of the Carma-RXR1 and Carma-RXR2 are shown. The nucleotide and amino acid residue numbers are indicated at the both sides of each lane. Bold underlining indicates RXR primers. Down vertical arrow indicates the -3 ∆D variant (amino acids TFQ) that is present in Carma-RXR1. Dotted underlines indicate +42 ∆E variant that is absent in Carma-RXR1. *Indicates the stop codon. In the 3' UTR, four mRNA recognition signals for degradative processing (ATTAA) are shown in solid boxes. The putative polyadenylation signals (A)n are shaded. Micro satellite repeats (CT)n are underlined.

**Figure 1** Nucleotide and deduced amino acid full-length sequence of the Carma-RXR1 and Carma-RXR2. The nucleotide and amino acid residue numbers are indicated at the both sides of each lane. Bold underlining indicates RXR primers. Down vertical arrow indicates the -3 ∆D variant (amino acids TFQ) that is present in Carma-RXR1. Dotted underlines indicate +42 ∆E variant that is absent in Carma-RXR1. *Indicates the stop codon. In the 3' UTR, four mRNA recognition signals for degradative processing (ATTAA) are shown in solid boxes. The putative polyadenylation signals (A)n are shaded. Micro satellite repeats (CT)n are underlined.
then dissected as above to remove the OV and some of the HP. The OV was calculated for each experimental crab (Nagaraju et al. 2004).

**Quantitative real-time PCR**

cDNA was produced from total RNA extracted from the tissues (e.g. HP, ovary, and other tissues) of green crabs in different reproductive stages (based on the color and OI; Nagaraju et al. 2004). The cDNA was incubated with RXR GSPs (CmRXR-F1 and CmRXR-R3) that produce a 143 bp product and Vg GSPs (CmVg-F1 and CmVg-R1) produced a 184 bp product (Ding et al. 2010). For a reference gene, we used AK (GenBank accession number AF167313) using the primers CmAK-F1 and CmAK-R1 which produce a 319 bp product. For qPCR, 1 μg of cDNA was amplified with 0.2 μmol of these GSPs in a 30 μl reaction containing Sybr Green Master Mix (Applied Biosystems). Methods and calculations used for the qPCR analysis of the RXR and Vg messengers have been described previously (Ding et al. 2010).

**Quantification of MF levels in hemolymph by HPLC**

Hemolymph levels of MF were determined by a HPLC method that has been previously described and validated for *C. maenas* (Borst & Tsukimura 1991). Briefly, hemolymph (0.5 ml unless otherwise noted) was withdrawn through the arthroial membrane of the coxa of a walking leg. It was added to 2.5 ml acetonitrile and 4% NaCl to give a total volume of 4.5 ml. The mixture was extracted with 1.5 ml hexane containing 20 ng of cis, trans-MF (used as an internal standard) and the hexane supernatant was analyzed by normal-phase HPLC (silica column; 1% diethyl ether in hexane; u.v.; 220 nm). We calculated the MF content of each sample by comparing its MF peak area with that of an MF standard. The lower limit of MF that was detectable by this method was 2 ng/ml.

**Statistical analysis**

The data were analyzed using Instat Software (GraphPad, San Diego, CA, USA). Data with multiple groups were analyzed by one-way ANOVA followed by the Student–Newman–Keuls test to determine significance.

**Results**

**Molecular cloning and sequence of full-length RXR**

PCR-based cloning methods were used to obtain the spliced variants of cDNA for ovarian RXR. A pair of primers (CmRXR-F1, CmRXR-R1; Table 1) was synthesized using the partial sequences for green crab RXR in GenBank. The primers amplified a 488 bp product from a cDNA derived from ovarian total RNA. The PCR product was cloned, and its sequence was identical to those of the partial RXR sequences in GenBank (Towle & smith 2006). We then synthesized GSP primers for 3′ and 5′ RACE amplification. CmRXR-F2 and the UPM amplified a 1235 bp cDNA from ovarian RNA, which was identical (100%) to the known partial RXR gene sequences of *C. maenas*. The 3′ RACE sequence was used to design the CmRXR_R2 primer that was used with the UPM in a 5′ RACE. Two 5′ RACE products (781 and 898 bp) were obtained, cloned and sequenced. The two sequences had a high degree of similarity and overlapped part of the 3′ RACE product. Together, these gave the full-length cDNAs for Carma_RXR1 (1796 bp) and Carma_RXR2 (1913 bp; Fig. 1). Analysis of the cDNAs showed that the open reading frame (ORF) for both RXR1 and RXR2 starts with an ATG codon at position 156 bp. For Carma_RXR1, the ORF ends at position 1362. This yields an ORF (1206 bp) that encodes a 402 amino acid protein. For Carma_RXR2, the ORF ends with a stop codon (TAG) at position 1479. This yields an ORF (1323 bp) that encodes a 441 amino acid protein. For confirmation of sequence, we amplified the ORF region of both RXR genes by UTR primer sets (CmRXR_F4 (UTR) and CmRXR_R4 (UTR); Table 1). The amino acid sequence of Carma_RXRs has two variant sequences occurred at two alternative splicing sites, one in the ‘T-box’ (tripeptide, TFQ) in the linker D domain and one (deletion of 42 amino acid residues) in the LBD (Fig. 2).

Amino acid sequence alignments of the C and E domains showed high levels of amino acid similarity to other crustaceans. The full ORF of Carma_RXR1 showed 89% identity with Carma_RXR2, 88% identity with *Gecarcinus lateralis* (G. lateralis) RXRα (AAZ20368), 92% identity with *U. pugilator* (AAC32789), and 71% identity with *Marsupenaeus japonicus* RXR (BAF75376; Fig. 2). The C domain in Carma RXRs includes cysteines that can form two zinc fingers for DNA binding. Amino acid sequences in the P-, T-, and D-boxes of Carma_RXRs are the same as those found in other RXRs (Fig. 2). The E/F domains show differences between Carma_RXRs and other RXRs but amino acid residues in the helices (H2–H13) connected to ligand binding are all conserved (Fig. 2).

The relationship of the Carma_RXRs to the RXRs of a few crustaceans is shown in Fig. 2. The A/B domains of nuclear receptors are transactivation domains and show the most variability between different receptors and between different isoforms of receptors often due to differential splicing in this region. The sequences shown in Fig. 2 are actually very similar to the other two brachyuran sequences previously reported with the exception of the extreme N-terminus. The C domain of Carma_RXR showed ~99% similarity to the other crustaceans. The hinge area is also highly similar to the other RXRs, thus indicating that Carma_RXR may recognize similar target sequences. The E/F region of Carma_RXR shows 60% similarity to RXRs of other crustaceans.

www.endocrinology-journals.org
Figure 2 Comparison of deduced amino acid sequences of full-length RXR cDNAs from crustaceans. Amino acid sequence of green crab RXR (Carma_RXR1 and Carma_RXR2) was aligned with RXRs from Gecla_RXRa, Celpu_RXR, and Marja_RXR using ClustalW. Dashed lines indicate gaps for optimizing alignment. Amino acid residues that are identical or similar between all the sequences are highlighted with symbols (*, single, fully conserved residue; :, conservation of strong groups; ., conservation of weak groups). There was a high degree of sequence identity in the DNA- (domain C) and ligand-binding (domain E) domains. Cysteine residues (asterisks) and the P- and D-box sequences in the C2C2 zinc-finger DNA-binding motif were conserved. Core sequence of the AF-2 domain near the N-terminus, is boxed. ▲ or ▼ indicate boundaries of each functional domain.
We measured the relative expression of RXR mRNA during the vitellogenesis using qPCR. GSPs for RXR mRNA were designed to amplify a 143 bp region of the C and D domains of the RXR cDNA. As a normalization process, we used a set of GSPs to amplify a 319 bp region of the AK cDNA. We found that ovarian levels of RXR mRNA were low in previtellogenic stage and were increased significantly \((P<0.001)\) from vitellogenic stages I to II followed by a significant \((P<0.001)\) decrease in vitellogenic stage III (Fig. 3). Changes in RXR mRNA levels had a different pattern in HP. Low levels of RXR mRNA were found in the previtellogenic, and significantly \((P<0.001)\) increased from vitellogenic stages I to III (Fig. 3). We also measured the relative expression of Vg mRNA during the vitellogenic cycle using qPCR (Ding et al. 2010).

**The effect of RXR-dsRNA and MF on the levels of RXR and Vg mRNA in ovarian and HP slices incubated in vitro**

Incubation of ovarian or HP slices with RXR-dsRNA caused a significant inhibition of RXR in both the ovary and the HP of green crab. Levels of Vg mRNA were also significantly \((P<0.001)\) lowered by this treatment. RXR levels in dsRXR-treated ovary and HP slices were three and 14-fold lower, respectively, than in the corresponding control slices (Fig. 4A and B). Treatment of ovary and HP slices with MF caused a significant \((P<0.001)\) increase (three and 50-fold, respectively) in RXR mRNA and Vg mRNA. No significant change was observed either in RXR or in Vg mRNA levels between the initial and the concurrent controls (GFP-treated) in either ovary or HP fragments (Fig. 4A and B).

In vivo effect of MF and RXR-dsRNA on ovarian maturation

Injection of MF significantly \((P<0.001)\) increased the OI (over 50%), compared with the corresponding values for the control (Fig. 5). No significant change in OI was observed for crabs treated with RXR-dsRNA plus MF compared with
the concurrent control (GFP treated) crabs (Fig. 5). All MF-injected crabs entered into vitellogenic stage II, whereas crabs treated with RXR-dsRNA were in vitellogenic stage I. Interestingly, the ovarian morphology of crabs treated with RXR-dsRNA was pale yellow color, and the tissue appeared to be decaying. This effect may be due to significant inhibition of RXR and Vg levels in the ovary and HP.

In vivo effect of MF and RXR-dsRNA on Vg and RXR transcript levels in ovary and HP of green crab

Injection of dsRNA (Carma_RXR) caused significant ($P<0.001$) inhibition of RXR messenger (ninefold for HP and tenfold for ovary; Fig. 6A and B) levels compared with the corresponding values of the concurrent control (GFP injected crabs) in both HP and ovary of green crab. Levels of Vg mRNA were also significantly ($P<0.001$) lowered by this treatment. Treatment of female crabs with MF caused a significant ($P<0.001$) increase (twofold RXR and threefold Vg in ovary and 0.4-fold Vg and twofold RXR in HP respectively) in RXR mRNA and Vg mRNA. No significant change was observed either in RXR or in Vg mRNA levels between the initial and the concurrent controls (GFP injected) in either ovary or HP fragments (Fig. 6A and B). Treatment of female crabs with both MF and dsRNA (Carma_RXR) caused significant ($P<0.001$) inhibition of RXR and Vg messenger levels compared with the corresponding values of the control in both HP and ovary of green crab (Fig. 6A and B).

In vivo effect of RXR-dsRNA on MF levels in hemolymph

For the in vivo experiment, we tested the effects of dsRNA (Carma_RXR) on the MF levels of female green crabs. The dsRNA (Carma_RXR) significantly ($P<0.001$) decreased MF levels in female green crabs (Fig. 7). These results indicate that RXR can affect MF production and is also involved in ovarian development in *C. maenas* (Fig. 5).

**Discussion**

Identification of two different RXRs in the ovary of green crab by cloning of the RXR genes provides an opportunity to identify the expression levels in different vitellogenic stages.
of crabs using RT-PCR, and qPCR analysis. Considering the expression levels of the transcript, we propose that the two RXRs may be involved in the reproductive functions in female green crabs. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number EU683888 (Carma_RXR1) and EU683889 (Carma_RXR2).

To date, the isoforms of crustacean RXR were identified from G. lateralis, Celuca pugilator (C. pugilator), M. japonicus, Fenneropenaeus chinensis (F. chinensis), and Daphnia magna (Durica & Hopkins 1996, Chung et al. 1998a,b, Kim et al. 2005, Asazuma et al. 2007, Wang et al. 2007, Priya et al. 2009). The amino acid sequences for the Carma_RXRs show significant similarity with RXR of crustaceans suggesting harmony of ligand-binding distinctiveness and function of the receptor in these phylogenetically divergent crustacean species (Fig. 2). All the identified RXR homologs have similar domains, but they vary in the association of those domains (Bonneton et al. 2003). RXR homologs have deduced LBD sequences that are more similar to the invertebrates, such as locusts (Hayward et al. 1999), ticks (Guo et al. 1998), cockroaches (Maestro et al. 2005), and vertebrate RXR (data not shown). RXRα (human) is well conserved in that of crustacean RXR’s, implying that crustacean RXRs might be more similar to vertebrate RXR than to insect USP in ligand binding (Billas et al. 2001, Carmichael et al. 2005, Nagaraju et al. 2010).

The distinctiveness of RXR is the existence of more variants in crustaceans than in insects (Wu et al. 2004, Kim et al. 2005, Priya et al. 2009). The major difference seems to be the inability to detect A/B domain variants in brachyuran RXRs, as observed in vertebrates and some insects. In this study, the sequence has identified variants in both the hinge and LBD (Figs 1 and 2), similar to that observed in other brachyuran crabs. This could lead to other splicing combinations. For example, the RXR splicing combinations have been verified in Uca so that all possible combinations (+/− in both regions) are observed (Wu et al. 2004). Furthermore, splicing variants in the RXR LBD have been identified in a few crustacean species such as six spliced variants in C. pugilator (Durica et al. 2002), nine spliced variants in G. lateralis (Kim et al. 2005) two spliced variants in F. chinensis (Priya et al. 2009), and one in M. japonicus (Asazuma et al. 2007). Both Carma_RXR1 and M. japonicus RXR have 42 amino acid deletions at the LBD site (Figs 1 and 2; Asazuma et al. 2007). Furthermore, Durica et al. (2002) observed two Celpu_RXR isoforms, termed T (+5) and T (−5), differing in a five amino acid (VGAVE) sequence within the T-box. Kim et al. (2005) reported on the Gecla_RXR, T (+8) variant within the T-box. Similarly, we also observed the two Carma_RXR isoforms T (+3) and T (−3) within the T-box.

We also found that ovarian levels of RXR mRNA were low in previtellogenic stage, increasing three- to eightfold from vitellogenic stages I to II followed by a significant decrease (P<0.001) in vitellogenic stage III (compared with previtellogenic stage; Fig. 3). These results indicate that RXR is important to early ovarian maturation, probably in orchestration of sex determination. Similarly, a threefold
increase can be observed during ovarian maturation in Uca for RXR (Durica et al. 2002). In daphnia, stage I embryos exhibited elevated RXR transcript levels relative to all subsequent embryonic stages. These changes in RXR mRNA levels had a different pattern in HP. Low levels of RXR mRNA were found in the previtellogenic, and significantly (90-fold) increased from vitellogenic stages I to III (Fig. 3). Similarly, we observed the relative expression of Vg mRNA during the vitellogenesis using qPCR (Ding et al. 2010). Taken together, these observations indicate that RXR stimulates Vg synthesis in HP for ovarian maturation.

RXR has been identified for which terpenoids can serve as ligands. JH has been inferred as ligands for the insect RXR orthologs (Wozniak et al. 2004). Recent studies suggest that MF itself may be a functional signaling molecule in Drosophila via high-affinity interaction with RXR (Jones & Jones unpublished data). In the present in vivo and in vitro investigation, MF stimulated RXR as well as Vg transcript levels in both HP and ovary of green crab (Figs 4 and 6). Similarly, elevated daphnia RXR mRNA levels early in embryo development co-occur with elevated ecdysteroid levels, which may indicate induction of RXR by ecdysteroids. Furthermore, Tamone & Chang (1993) reported that MF stimulates ecdysteroid secretion from crab Y-organs in vitro. These studies potentially indicate that MF is the upstream signaling molecule of ecdysteroid and RXR. Also, MF is required for both RXR and Vg synthesis at the transcript level. However, Hopkins et al. (2008) suggest that MF itself may be a functional signaling molecule in crustaceans via high-affinity interaction with RXR. Future studies may reveal ligand-binding similarities between insect and crustacean RXR.

Vitamin A derivatives such as RA can bind to two nuclear receptors such as RXR and RA receptors (RARs) to stimulate or inhibit gene transcription. RXR and RAR thus heterodimerize in the absence of ligand and are then bound to retinoid X response elements on the DNA. Agonist RA binds to RXR and the dimer undergoes a conformational change resulting in detachment of co-repressors and recruited co-stimulators. Co-stimulators can then bind to the RXR complex, which may help to relax the chromatin structure from the histones or may cooperate with the transcriptional pathway (Bouton et al. 2005). The steroid and terpenoid compounds can up- or downregulate the expression of target genes (Combs 2008). For example, EcR–RXR DNA-binding activity is stimulated by either 9-13 isomers of RA or ecdysteroid, demonstrating that hormones can play a role in heterodimer stabilization (Thomas et al. 1993). Exogenous retinoids stimulated total Celpu_RXR mRNA expression in crab limb blastemas (Chung et al. 1998a,b) and disrupted normal regeneration when applied at the time that the blastema was first forming (Hopkins & Durica 1995). Consistent with these observations, exogenous MF increased RXR transcript levels and stimulated the OI in female green crabs (Figs 5 and 6). Furthermore, dsRNA-RXR treatment significantly reduced the RXR transcript levels as well as MF levels in the hemolymph of green crab (Figs 6 and 7). Taken together, these results indicate that both RXR and MF are essential for ovarian maturation and RXR appears to act as a modulator for the MF signaling pathway.

RAs, either cis or trans forms, are morphogens. Associated but unusual compounds, such as JHs in insects and MF in crustaceans, exhibit similar functions (Gilbert et al. 2000). MF is established as a major morphogen in crustaceans (Laufer et al. 2005, Nagaraju & Borst 2008). MF is a key endocrine controller of crustacean molting, reproduction, osmoregulation, behavior, morphogenesis, and metabolism (see reviews Nagaraju (2007, 2011)). MF is found in the hemolymph of several crustacean species and is produced by the MO. MF is the major gonadotropic hormone stimulating the gonad development and the maturation of sexual behavior (reviewed by Nagaraju (2007)). In the present in vivo and in vitro study, the female green crabs, which received ME entered into the vitellogenic stage and also significantly increased the Vg and RXR messenger levels in MF-treated ovary and HP of green crabs (Figs 4 and 6). These results indicate that MF and RXR might have similar functions and are interdependent on one another. In addition, previous literature demonstrates that MF stimulates ovarian maturation and molting (Nagaraju et al. 2003, Reddy et al. 2004), and the JH III analog, methoprene, can stimulate mammalian RXR (Harmon et al. 1995). A few speculations are made, i.e.

1) There is a strong functional interaction between the MF and the RXR.
2) RXR may be interacting with EcR by stimulatory action of MF through Y-organ. This EcR–RXR complex may induce ovarian development.
3) EcR–RXR complex may bind with MF and form a heterotrimeric complex (MF–RXR–EcR) and this complex may stimulate ovarian maturation.
4) RXR may act as a modulator for the MF signaling pathway.

Conclusion

In summary, we have isolated two alternative spliced isoforms of Carma_RXRs from the ovary of the green crab, and the deduced amino acid sequence shows highest similarity to that of other crustacean species (Figs 1 and 2). Based on the amino acid sequence similarity, it can be considered a nuclear receptor family member. The expression levels of RXR messenger changes during vitellogenic stages of female green crab (Fig. 3). The RXR messenger levels are lowest in the vitellogenic stage III of the ovary as shown by qPCR. Furthermore, the relative expression of Vg transcript levels was changed during the reproductive cycle in female crabs (Ding et al. 2010). MF stimulates RXR and Vg messenger levels in vitro and in vivo (Figs 4, 6, and 8). Knockdown of RXR transcript levels significantly decreased the MF levels in hemolymph as well as the OI of green crab (Figs 5, 7, and 8). These results suggest that the RXR may be involved in the MF signaling pathway, or it may be interacting with EcR by stimulatory action of MF, or it may be interacting directly.
with MF. Finally, di- (MF–RXR or EcD–RXR) or trimeric (MF–RXR–EcD) complexes may induce ovarian maturation in these green crabs (Fig. 8).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgements

This research manuscript is dedicated to Prof. David W Borst, who passed away on September 27, 2010. His suggestions helped me a lot to improve this manuscript. He was a dedicated teacher, advisor, and a brilliant scientist who is missed by all who knew him. We thank the National Science Foundation (NSF; IBN-0611447 to Prof. D W B) for the financial support during my Postdoctoral investigation. We also thank four anonymous reviewers for critically reviewing the manuscript and Dr. Arif Ali and Dr. Christopher David Corso for their critical reading and suggestions.

References


www.endocrinology-journals.org
Low, S., Browne, M., Boudjelas, S. & De Poorter, M. 2000 100 of the World’s Worst Invasive Alien Species A selection from the Global Invasive Species Database, 12pp. Published by The Invasive Species Specialist Group (ISSG) a specialist group of the World Conservation Union (IUCN).


Received in final form 6 June 2011

Accepted 5 July 2011

Made available online as an Accepted Preprint 5 July 2011