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The brown shrimp (*Crangon crangon* L.) ecdysteroid receptor complex: Cloning, structural modeling of the ligand-binding domain and functional expression in an EcR-deficient *Drosophila* cell line

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ABSTRACT

cDNAs encoding ecdysteroid receptor (EcR) and retinoid X receptor (RXR) were cloned and sequenced from brown shrimp *Crangon crangon* (Crustacea: Decapoda), a common faunal species and commercially important in the North-West European coastal waters. A 3D model of the ligand-binding domain (LBD) of EcR was created and docking of ponasterone A (PonA) was simulated *in silico*. Finally, we report the transfection of expression plasmids for these receptors in the mutant *Drosophila* L57-3-11 cell line. Through an ecdysteroid responsive reporter assay we clearly prove the functionality of shrimp ecdysteroid receptor in the transfected L57-3-11 cell line. Our results indicate that the *Drosophila* L57-3-11 cell line and *in silico* LBD modeling can be used to study the function of crustacean ecdysteroid receptors and be applied to assess endocrine disrupting effects on non-target crustacean species.

1. Introduction

Ecdysteroids are known to play a crucial role in the coordination of development and reproduction in arthropods, nematodes and several other protostome clades. Ecdysteroids, of which 20-hydroxyecdysone (20E) is believed to be the most active form, trigger the specific regulation of several genes in different tissues and developmental stages, through interaction with the ecdysteroid receptor [EcR; see Hopkins (2009) for a recent review on crustacean ecdysteroids and EcR]. In order to bind to the ecdysteroid responsive elements (EcREs) in the promoter regions of ecdysteroid responsive genes, EcR needs to dimerize with the orphan retinoid X receptor (RXR). EcR and RXR belong to the superfamily of nuclear receptors (NR) and share NR-typical domain structures and gene regulatory mechanisms. NRs are characterized by a modular structure comprising five distinct structural and functional protein domains (Evans, 1988; Billas et al., 2009): (a) the A/B-domain, a highly variable N-terminal domain involved in transcriptional activation; (b) the C-domain, a highly conserved DNA-binding domain (DBD); (c) the D-domain, a flexible and variable hinge region involved in EcRE recognition (A-box) and heterodimerization (T-box); (d) the E-domain, a rather complex ligand-binding domain (LBD) consisting of 12 α-helices and two β-sheets with a complex tertiary structure subject to conformational changes which is involved in ligand binding, heterodimerization and interaction with other transcription factors; and finally (e) the C-terminal F domain: a highly variable domain of which the function is not well defined. NRs are categorized into seven (0–6) subfamilies, based on sequence homology (Nuclear Receptors Nomenclature Committee, 1999). EcR, the first member of group H of the NR subfamily 1, is related to Liver X receptor (LXR) and Farnesoid X receptor (FXR), two vertebrate steroid hormone receptors. RXR, the fourth member of group B of the NR subfamily 2, has been isolated in a wide variety of metazoans, where RXRs are common heterodimeric partners for many other NRs.

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The name RXR refers to 9-*cis* retinoic acid, the putative ligand in the vertebrate RXRs (Wolf, 2006), whereas the natural ligand(s) for invertebrate RXRs is still under debate. While EcR structure and functioning in insects (especially Diptera and Lepidoptera) is well documented, our understanding of EcR structure and functioning in crustaceans remains limited.

Here we report the molecular cloning and analysis of EcR and RXR of the brown shrimp, *Crangon crangon* L. (Crustacea; Decapoda; Caridea), a common faunal and commercially important crustacean species in the coastal waters of North-Western Europe. Subsequently, we present an *in silico* derived model of the LBD of CrcEcR bound to ecdysteroid. Finally, we report the transfection of expression plasmids of these receptors in a *Drosophila* cell line, and the use of this transfected cell line in an assay with an ecdysteroid responsive reporter construct to screen for ecdysteroid responsiveness of the shrimp EcR-RXR receptor complex. The mutant *Drosophila* Kc cell line L57-3-11 (Swevers et al., 1996; Cherbas and Cherbas, 1997; Hu et al., 2003) we use is characterized by inactivated DmEcRB1 and DmEcRB2 isoforms, which results in a 90% loss of endogenous 20E response and represents a continuous arthropod cell line which can be used to study ligand binding to exogenous (e.g. crustacean) EcRs.

2. Materials and methods

2.1. Collection of C. crangon and isolation of RNA

Ovaries were dissected out of a large (>75 mm total length) female *C. crangon* which was sampled during low tide at Bredene beach (Belgium). Total RNA was isolated through acid guanidinium-phenol–chloroform extraction (Chomczynski and Sacchi, 1987) using TriReagent® (Sigma–Aldrich NV, Bornem, Belgium), according to the manufacturer's protocol. The quality and quantity of the extracted RNA was examined by gel electrophoresis and spectrophotometry using a Nanodrop™ ND-1000 (Thermo Fisher Scientific BVBA, Asse, Belgium).

2.2. Cloning of CrcEcR and CrcRXR

First strand cDNA synthesis was performed using SuperScript™ II reverse transcriptase (Invitrogen NV, Merelbeke, Belgium) with the oligo(dT)$_{12-18}$ primers according to the manufacturer's protocol. The complete encoding cDNA sequences of CrcEcR and CrcRXR were derived using several successive PCR techniques. First, partial sequences for the DBDs were obtained through degenerate PCR (see Supplementary Table S1 in supplementary material for primer sequences) consisting of 35 cycles of 50 μl reactions (3 mM $MgCl_2$ and annealing temperature 59 °C). Degenerate primers were designed based on the known coding sequences from four other Decapoda species: *Carcinus maenaes*, *Gecarcinus lateralis*, *Geluca pugilator* and *Marsupenaeus japonicu* (see Supplementary Table S2 in supplementary material for the accession numbers of the used sequences). The PCR products were purified after electrophoresis using the QIAEX II gel extraction kit (Qiagen GmbH, Hilden, Germany), were ligated in pGEM®-T easy vector (Promega Benelux, Leiden, The Netherlands) and transformed in *Escherichia coli* TOP10F' cells (Invitrogen). After colony PCR, the plasmids from several positive colonies were purified using the Qiaprep Spin Miniprep kit (Qiagen) and sequenced by AGOWA GmbH (Berlin, Germany). Based on the obtained encoding cDNA sequences forward, non-degenerate primers in the DBD were designed and combined with a reverse degenerate primer situated in the ligand-binding domain (LBD). The same cloning routine was performed as described above. Subsequently, 5′ and 3′ RACE PCRs were performed using gene specific primers (GSPs) designed in the obtained DBD-LBD sequences. Initially, the SMART™ RACE cDNA amplification kit (Clontech Laboratories, Palo Alto, CA) was used with some slight alterations to the manufacturer's proposed reaction mix. Reactions included 1% (v/v) RACE-Ready cDNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.025 U/μl Platinum® Taq DNA Polymerase (Invitrogen) and 0.25 μM GSP in a total volume of 10 μl. Further nested PCR with inner primers on the undiluted primary RACE PCR products was needed to obtain a satisfactory yield, except for the 3′ cDNA end of CrcRXR. The RACE PCR for the 3′ cDNA end of CrcEcR had to be performed with the FirstChoice RLM RACE kit (Applied Biosystems, Lennik, Belgium) and included an extra nested PCR, using 2 mM $MgCl_2$, 0.2 mM dNTP mix and 0.025 U/μl Platinum® Taq Polymerase in 50 μl reactions. After cloning and sequencing, 5′ and 3′ end primers were designed to amplify and ligate the full open reading frames (ORFs) into the Ract-Hadh expression vector (Swevers et al., 1996). The Kozak sequences were slightly adapted to a *Drosophila* Kozak consensus sequence (Cavener, 1987), while restriction sites and some extra nucleotides were added to facilitate primer annealing (see Supplementary Table S1 in supplementary material for primer sequences). The PCR of the full ORFs was performed with the Expand Long Range dNTPack (Roche Applied Science, Mannheim, Germany) at an annealing temperature of 48 °C. Restriction fragments corresponding to CrcEcR and CrcRXR ORFs were purified with the E.Z.N.A.® Cycle Pure kit (OMEGA Bio-Tek Inc, Norcross, GA, USA), ligated with T4 DNA ligase (Promega) in the Ract-Hadh vector (Swevers et al., 1996), and transformed in *Escherichia coli* TOP10F' cells (Invitrogen). Positive clones were selected and grown in 200 ml cultures overnight and the plasmids were extracted with the E.Z.N.A.® plasmid Maxi kit (OMEGA Bio-Tek) to obtain high quality plasmid DNA necessary for transfection of L57-3-11 cells.

2.3. Sequence analysis and phylogenetic analysis

Secondary structure predictions of the LBDs were performed with the freely available neural network algorithms NNPredict (McClelland and Rumelhart, 1988; Kneller et al., 1990), Porter (Pollastri and McLysaght, 2005) and Prof (Ouali and King, 2000; Meiler et al., 2001; Meiler and Baker, 2003). The available EcR (and related deuterostome LXR and FXR) and RXR (including Diptera and Lepidoptera Ultraspiracle receptor, USP) sequences were obtained through a similarity search using the BLAST tool (http://www.blast.ncbi.nlm.nih.gov/blast.cgi) and reduced to 45 EcR/LXR and 52 RXR/USP sequences, which subsequently were aligned using ClustalW2 (Larkin et al., 2007). The phylogenetic trees of receptor LBDs were constructed with MEGA 4.1 (Tamura et al., 2007) using the neighbor-joining method (Saitou and Nei, 1987). Gaps in the alignment were handled with the complete deletion method and a bootstrap test with 1000 replications (Efron, 1979) was performed. The evolutionary distances were computed using the Poisson correction for amino acids (Zuckerkandl and Pauling, 1965).

2.4. 3D modeling of the ligand-binding pocket and docking of ponasterone A

Multiple amino acid sequence alignments were carried out with CLUSTAL-X (Thompson et al., 1997) using the Risler's structural matrix for homologous amino acid residues (Risler et al., 1998). Molecular modeling of the CrcEcR LBD was carried out on a Silicon Graphics O2 R10000 workstation, using the programs InsightII, Homology and Discover3 (Accelrys, San Diego CA). The atomic coordinates of *Tribolium castaneum* TcEcR LBD in complex with the ecdysteroid Ponasterone A (PonA; RCSB Protein Data Bank code 2NXX) (Iwema et al., 2007) were used to build the 3D model of the receptors. Steric conflicts were corrected during the model building procedure using the rotamer library (Ponder and Richards,

1987) and the search algorithm implemented in the Homology program (Mas et al., 1992) to maintain proper side-chain orientation. An energy minimization of the final model was carried out by 150 cycles of steepest descent using the cvff force field of Discover. PROCHECK (Laskowski et al., 1993) was used to assess the geometric quality of the 3D model. Molecular cartoons were drawn with PyMol (W.L. DeLano, http://www.pymol.sourceforge.net). The fold recognition program Phyre (http://www.sbg.bio.ic.ac.uk/phyre/html/index.html) (Bennett-Lovsey et al., 2008) that also used 2NXX and structurally related proteins as templates yielded readily superposable 3D models. TcEcR LBD in complex with PonA was taken as a template for docking PonA to CrcEcR LBD. Docking was performed with InsightII using Discover3 as a forcefield. Clipping planes of CrcEcR LBD complexed to ecdysone and tebufenozide were rendered with PyMol.

2.5. Transfection and growth conditions of EcR-deficient Drosophila Kc L57-3-11 cell line

The L57-3-11 cell line was maintained in Schneider's medium containing 10% heat inactivated fetal bovine serum (Invitrogen) and 1% antibiotic antimycotic solution (Sigma) at 25 °C under a constant 10 μM 20E selection to keep endogenous DmEcR expression low. The following transient transfections of the *Drosophila* cell line were performed with Lipofectin® Reagent (Invitrogen) according to the manufacturer's protocol with 1.5 μg of each plasmid per 3×10^6 cells in a 500 μl transfection reaction: (a) a positive control (*Drosophila melanogaster* DmEcRB1 isoform), (b) CrcEcR and CrcRXR combined and (c) a negative control (empty Ract-Hadh vector). With each transfection, 1.5 μg of EcRE-b.act.luc reporter plasmid (Soin et al., 2009) was co-transfected. The EcRE-b.act.luc reporter construct is composed of seven copies of the EcRE derived from the *Drosophila* hsp27 promoter, a *Bombyx mori* derived basal actin promoter (b.act) which is followed by the reporter gene for firefly luciferase (luc) and a termination signal. Starting at 3×10^6 cells, the transfected cells were grown for 72 h in a 6-well plate at 25 °C.

2.6. In vitro reporter assay with transfected Drosophila Kc L57-3-11 cell line

After transfection, EcR-deficient *Drosophila* cells were exposed in quadruplicate during 24 h at 25 °C to 10 nM PonA or 1 μM 20E in order to quantitate their ecdysteroid responsiveness. Luciferase expression was measured using the Steady-Glo® luciferase assay system kit (Promega, Leiden, The Netherlands) with a Tecan M200 luminometer (Tecan, Mechelen, Belgium). Stock solutions of 1 μM PonA (⩾95%; Invitrogen) and 100 μM 20E (⩾95%; Invitrogen) were prepared in ethanol.

In order to confirm the high expression levels of the transfected CrcEcR and CrcRXR plasmids in comparison with the endogenous DmEcR, semi-quantitative RT-PCR experiments were performed. RNA was extracted from approximately 6×10^6 control Kc L57-3-11 cells and Kc L57-3-11 cells transfected with CrcEcR and CrcRXR. Prior to the cDNA synthesis, a RQ1 RNase-free DNase (Promega) treatment was applied using 100 μg/ml RNA template. cDNA was synthesized as described above using a final concentration of 10 ng/μl random hexamer primers. For the RT-PCR, reactions were performed containing 0.5% (v/v) cDNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.025 U/μl Platinum® Taq DNA Polymerase (Invitrogen) and 0.25 μM GSPs at a final volume of 10 μl. Specific primer pairs were designed for CrcEcR (5′-CGG AAA TAA CTG CGA AAT GGA-3′ and 5′-CCT GAC AAT CTC GTC ATA GGC-3′), CrcRXR (5′-GCA CTG TGC GAA AAG ACC TCA-3′ and 5′-TTT GCC CAT TCT ACA AGT TGC-3′), DmEcR (5′-CCT CCG GCT ACC ACT AC AAC-3′ and 5′-GGC TCG CAT GTC ATA AGG TC-3′) and DmUSP (5′-ATA GAC AAG CGG CAG AGG AA-3′ and 5′-GGA CTG TGG AAT AGG GAC CA-3′) which yield PCR products of ~360–380 bp for each primer pair. As for the external control, universal 18S rRNA primers were designed (5′-CCC GTC GCT ACT ACC GAT T-3′ and 5′-GAT CCT TCC GCA GGT TCA C-3′). Following PCR conditions were used: an initial 30 s denaturation at 94 °C, followed by 35 cycles of 30 s denaturation at 94 °C, annealing at 55 °C and 30 s elongation at 72 °C. The RT-PCR experiment consisted of triplicate reactions sampled at the end of the annealing step after 23–26–29 cycles for 18S rRNA, 29–32-35 cycles for CrcEcR and CrcRXR and 35–38–41 cycles for DmEcR and DmUSP. All PCR products were visualized on a single 2% agarose gel by ethidium bromide staining. Relative intensity of each band was determined through ImageJ image processing program (Abramoff et al., 2004).

3. Results

3.1. CrcEcR structure

Initially, degenerate PCR yielded two short partial sequences of the strongly conserved DBD (79 bp) and LBD (168 bp). The use of a non-degenerate primer pair, targeting the DBD and LBD, allowed determination of four longer identical sequences (from DBD to LBD) of 778 bp, allowing the development of gene specific primers for RACE PCR. A 442 bp and two 403 bp identical 5′ RACE cDNA clones and two identical 501 bp 3′ RACE cDNAs were isolated. The full open reading frame (ORF) cDNA sequence of 1323 bp was generated using primers designed at the start and stop codon. CrcEcR showed high similarity to EcR of other crustacean species *C. pugilator* and *M. japonicus* with 77% and 67% similarity over the entire length, respectively (Table 1). Compared to similar EcRs, CrcEcR contains a short A/B-domain of merely 25 amino acids (AAs), which appear to be strongly conserved within Decapoda (Fig. 1A). The DBD domain AA sequence of CrcEcR is identical to that of the crab *C. pugilator*. The poorly conserved hinge region of CrcEcR is long compared to other Decapoda species and contains a predicted short α-helix (EEEGRQL) (Fig. 1A).

3.2. CrcRXR structure

Initially, a single clone of a partial DBD (84 bp) sequence was obtained through degenerate PCR. A 663 bp clone from DBD to LBD was sequenced using a degenerate reverse primer situated in the LBD. Two 591 bp and 653 bp cloned 5′ RACE PCR products exhibited different 5′ untranslated regions (UTRs), the three 3′ RACE PCR products (844 bp, 863 bp and 929 bp) were identical in the overlapping sequences. While in frame stop codons were detected in all clones, none of the sequenced 3′ UTRs contained a poly-A tail. Sequencing of the full ORF of 1218 bp shows 78% and 80% similarity to the RXR of *C. pugilator* and *M. japonicus*, respectively (Table 1). The A/B domain of CrcRXR is short but well conserved compared to the related species, particularly the 5′ sequence MSGSLDRQSPL and 3′ sequence LSTSP(S/T)QYPP(N/S). Minor differences can be observed in the DBD of CrcRXR compared to *C. pugilator*: Glu145 is replaced by Asp116, Ser147 is substituted by Ala118, and Thr168 by Gly139 (Fig. 1B). The 32 AA long hinge region is almost identical to CpRXR, only differing in two AAs. Based on secondary structure predictions the LBD appears to lack the second α-helix, a phenomenon also observed in RXR of other crustacean (Asazuma et al., 2007) and non-crustacean species (Hayward et al., 1999) (Fig. 1B).

3.3. Phylogenetic analysis based on EcR and RXR LBDs

The phylogenetic trees obtained through a neighbor-joining analysis using the LBD of EcR and USP/RXR are shown in Fig. 2.

Table 1
Comparison of predicted amino acid sequences of EcR and RXR receptor homologues with CrcEcR and CrcRXR.

Region	Length, in amino acids (similarity)								
	CrcEcR	CpEcR	MjEcR	DamEcR	BgEcR	AamEcR	DmEcR	NiEcR	HsLXR
A/B	32	156 (81%)	104 (71%)	326 (78%)	207 (68%)	188 (62%)	234 (65%)	209 (46%)	97 (18%)
C	66	66 (100%)	66 (98%)	66 (93%)	66 (96%)	66 (95%)	66 (89%)	66 (83%)	66 (69%)
D	117	74 (67%)	104 (54%)	109 (37%)	75 (49%)	94 (37%)	101 (29%)	77 (40%)	57 (19%)
E	218	218 (86%)	221 (69%)	217 (74%)	219 (73%)	219 (67%)	221 (59%)	217 (48%)	224 (40%)
F	7	4 (50%)	4 (-)	3 (33%)	3 (-)	3 (33%)	227 (14%)	1 (-)	3 (33%)
Total	440	518 (75%)	499 (67%)	721 (65%)	570 (64%)	570 (60%)	849 (54%)	570 (45%)	447 (37%)
	CrcRXR	CpRXR	MjRXR	DamRXR	BgRXR	AamRXR	DmUSP	CfiRXR	HsRXRα
A/B	76	105 (65%)	114 (73%)	81 (38%)	94 (42%)	81 (31%)	103 (28%)	80 (26%)	134 (22%)
C	66	66 (95%)	66 (95%)	66 (95%)	66 (92%)	66 (90%)	66 (92%)	66 (86%)	66 (86%)
D	31	26 (73%)	30 (63%)	25 (20%)	25 (28%)	36 (25%)	64 (29%)	29 (27%)	25 (24%)
E/F	232	268 (86%)	232 (83%)	228 (71%)	228 (73%)	231 (64%)	275 (43%)	230 (56%)	237 (68%)
Total	405	465 (83%)	442 (82%)	400 (66%)	413 (65%)	414 (60%)	508 (49%)	405 (53%)	462 (60%)

Amino acid length and similarity (between brackets) with CrcEcR and CrcRXR are given for *Celuca pugilator, Marsupenaeus japonicus, Daphnia magna, Blatella germanica, Amblyomma americanum, Drosophila melanogaster, Neomysis integer, Calanus finMarchicus and Homo sapiens* homologues. See Supplementary Table S2 in supplementary material for the accession numbers of the used sequences. CfiRXR was used as the available NiRXR sequence lacks the C-terminal 11 residues of the E/F-domain. Domain structures were defined based on conserved motif structure after alignment using ClustalW.

As these phylogenetic trees are based on amino acid substitutions within the LBD, sequence divergence may reflect changes in ligand-binding preferences. Phylogenetic relationships within the crustacean order of Decapoda are clearly visible in both trees exhibiting strong bootstrap values (Fig. 2A and B): the infraorder sister groups Brachyura (crabs; *Carcinus, Gecarcinus, Celuca*) and Caridea (shrimps; *Crangon*) are representatives of the suborder Pleocyemata, which in turn is the sister group of the suborder Dendrobranchiata (prawns; *Litopeneaus*). These two belong to the order Decapoda (superorder Eucarida) which together with the order Mysida (superorder Peracarida) are representatives of the class Malacostraca. The remarkable divergence of the *Neomysis integer* (Peracarida) EcR sequence used probably reflects a strongly divergent ligand-binding specificity. As no other Peracarida EcR and RXR sequences are currently available, it is unclear at what phylogenetic level this differentiation occurs. In our analysis, EcR and RXR of *Daphnia* (order Cladocera; class Branchiopoda) clusters with the subphylum Hexapoda, which, in case of the RXR tree, encompasses the divergent USP Lepidoptera and Diptera homologues. Both EcR and RXR phylogenetic relationships confirm earlier observations (Budd and Telford, 2009) that insects can be considered as a terrestrial (Pan) crustacean clade. Arthropod RXR/USP clearly separate from the other Protostomia clade of Mollusca RXR, which clusters closely with Deuterostomia RXRs. This has been observed before (Wang et al., 2007) and may be related to a common high affinity of 9-cis retinoic acid to Mollusca and Deuterostomia RXR (Mark and Chambon, 2003; Nishikawa et al., 2003).

3.4. Modeling of the ligand-binding pocket of CrcEcR and docking of PonA

The high percentages of both identity (~69%) and similarity (~91%) that CrcEcR LBD shares with the template TcEcR LBD (Mouillet et al., 1997) allowed us to build a rather accurate 3D model. About 87% of the residues of the modeled CrcEcR LBD were correctly assigned on the best allowed regions of the Ramachandran plot, the remaining residues being located in the generously allowed regions of the plot except for Asp35 which occurs in the non-allowed region (result not shown). Some discrepancies that were observed with our lab-made modeled structures essentially deal with the shape of the loops connecting the α-helical stretches of CrcEcR LBD. These discrepancies occur outside the groove responsible for the binding of ecdysteroid hormone. The modeled CrcEcR LBD consists of a canonical EcR LBD structure built up by 12 α-helices tightly packed around a ligand-binding groove that specifically anchors ecdysteroids (Fig. 3A). In addition, Fig. 3B demonstrates that the amino acid residues Glu224, Met254, Thr255, Gly310 and Tyr320 of the ecdysteroid-binding cavity create a network of 9 H-bonds participating in the binding of PonA in association with stacking interactions of residues Phe309, Tyr315 and Trp436. Upon docking to the ecdysteroid-binding cavity of CrcEcR LBD, the alkyl chain of PonA becomes inserted in one of the two pockets located at the bottom of the binding cavity (Fig. 3C). Compared with coleopteran EcR (Iwema et al., 2007), differences in steric interactions of PonA within the LBD appear to be limited to the stacking interaction of Tyr315 and the lack of a water-mediated binding of Asn410 at the end of the PonA hydrocarbon tail.

3.5. In vitro reporter assay with transfected Drosophila Kc L57-3-11 cell line

Semi-quantitative RT-PCR on the transfected L57-3-11 cell line clearly confirms that co-transfected CrcEcR and CrcRXR are overexpressed compared to the endogenous DmEcR and DmUSP mRNAs (Fig. 4). Inductions of the ecdysteroid responsive reporter gene in the CrcEcR/RXR and the DmEcR rescued cell line were similar (Fig. 5); after transfection with CrcEcR/RXR plasmid, reporter inductions are 4.09 ± 0.22 (1 μM 20E) and 3.71 ± 0.12 (10 nM PonA) times higher than the inductions in the control cell line containing empty vector. After transfection with DmEcR, these fold inductions are 4.14 ± 0.12 and 4.53 ± 0.17 for 1 μM 20E and 10 nM PonA exposure, respectively. For 1 μM 20E and 10 nm PonA exposure the measured RLU's were significantly different (*t*-test: $df = 6$, $n = 4$, $p < 0.01$) between the DmEcR/ CrcEcR-CrcRXR and Ract-Hadh transfections.

4. Discussion

In the present study, we cloned and characterized cDNAs encoding EcR and RXR from *C. crangon*. CrcEcR and CrcRXR exhibit the strongly conserved domain structure which is typical for NRs: a poorly conserved A/B transactivating domain, a strongly conserved DNA-binding C-domain, a variable hinge D-region, a well conserved ligand-binding E-domain and an extremely short F-tail which is even lacking in crustacean RXR (Chung et al., 1998; Durica et al., 2002; Wu et al., 2004; Kim et al., 2005; Asazuma et al., 2007; Wang et al., 2007; Hopkins et al., 2008). Based on the high sequence similarities with CpEcR (75%) and CpRXR (83%) of the closely related land crab *Celuca pugilator*, we can assume the

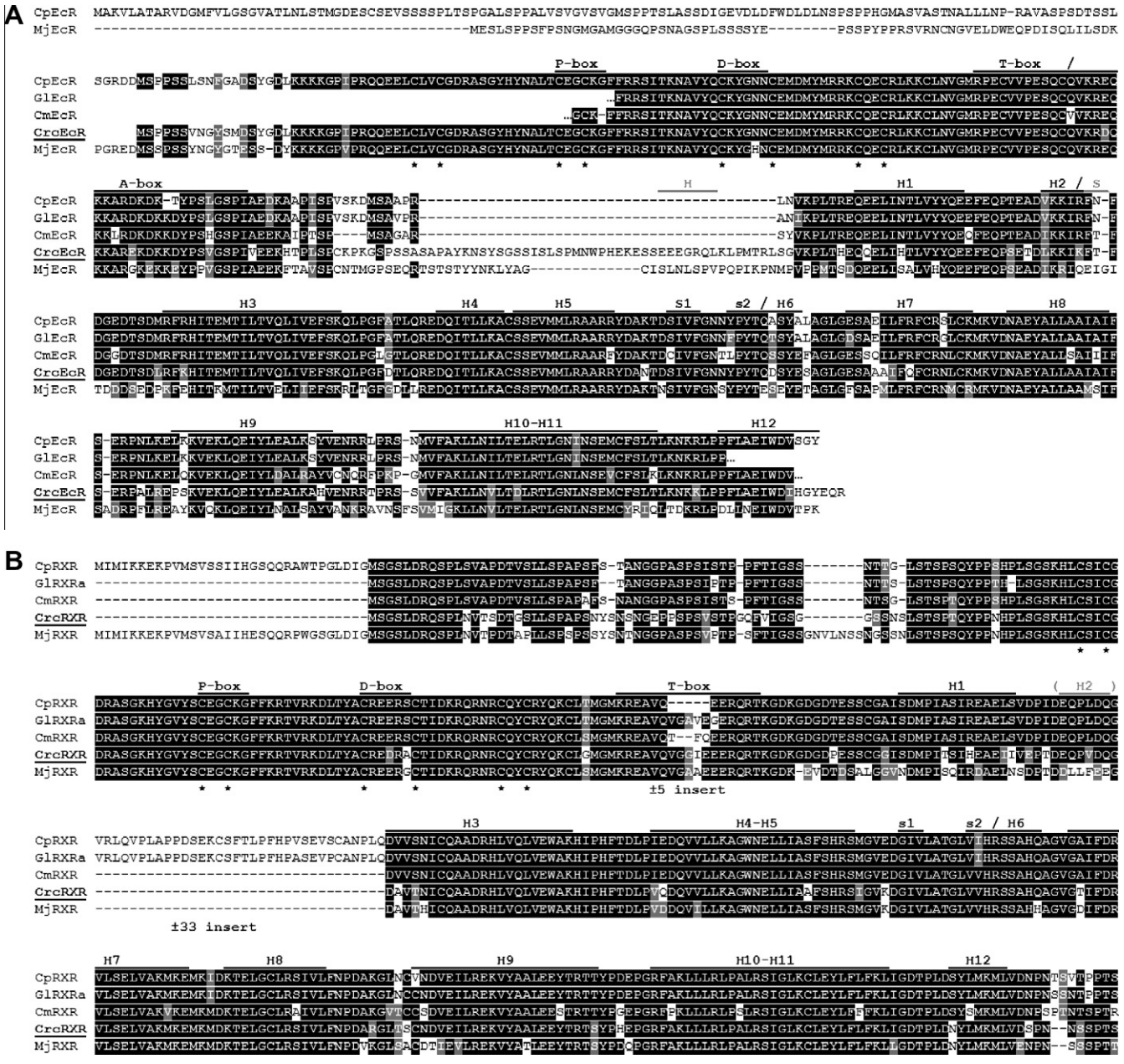

Fig. 1. Alignment of the CrcEcR (A) and CrcRXR (B) with the available EcR and RXR sequences of related Decapoda species. Alignment of the full length CrcEcR and CrcRXR ORFs cloned with the available EcR and RXR sequences of other Decapoda species; *Celuca pugilator*, *Gecarcinus lateralis*, *Carcinus maenas* and *Marsupenaeus japonicus*. Conserved α-helices (H1–H12), β-sheets (s1-s2) and domains are indicated above the sequences; conserved cysteine residues in the DBD and LBD and variant inserts are indicated below the sequences in Fig. 1AB. See Supplementary Table S2 in supplementary material for the accession numbers of the used sequences. The multiple aligned sequences were shaded using BOXshade 3.2 (http://www.ch.embnet.org/software/box_form.html).

successful cloning of brown shrimp CrcEcR and CrcRXR. The most striking sequential differences are present in CrcEcR, where the A/B-domain is limited to a strongly conserved stretch of 25 AAs and the hinge region has a C-terminal extent of 42 AA compared to the closely related Brachyura EcRs.

We used the obtained sequence data at first to *in silico* reconstruct the LBD of CrcEcR and predict the steric interactions involved in PonA docking, and in second to investigate the activity of the brown shrimp ecdysteroid receptor *in vitro* in the presence of PonA through a luciferase reporter system in the EcR-deficient *Drosophila* L57-3-11 cell line. The accurate 3D model indicates that the steric interactions involved in PonA docking are similar to that of coleopteran EcRs, as differences appear to be limited to the stacking interaction of Tyr315 and the lack of a water-mediated binding at the end of the PonA hydrocarbon tail. However, a water molecule was also not observed in the structures of the *Heliothis* and *Bemisia* ecdysteroid receptors complexed with PonA (Billas et al., 2003; Carmichael et al., 2005).

Subsequently, the *in vitro* EcR reporter assay clearly indicates the activity of the CrcEcR receptor in the mutant *Drosophila* L57-3-11 cell line: binding of 1 μM 20E and 10 nM PonA to transfected shrimp CrcEcR/RXR led to fourfold increases in EcR dependent reporter activation, comparable to the increase after 'rescue' transfection with fly DmEcR. In similar experiments with the L57-3-11 cell line with exogenous lepidopteran EcR (Swevers et al., 1996), induction in the BmEcR expressing cell line was much lower than in the DmEcR rescued cell line. These researchers proposed that higher exogenous EcR concentrations are required

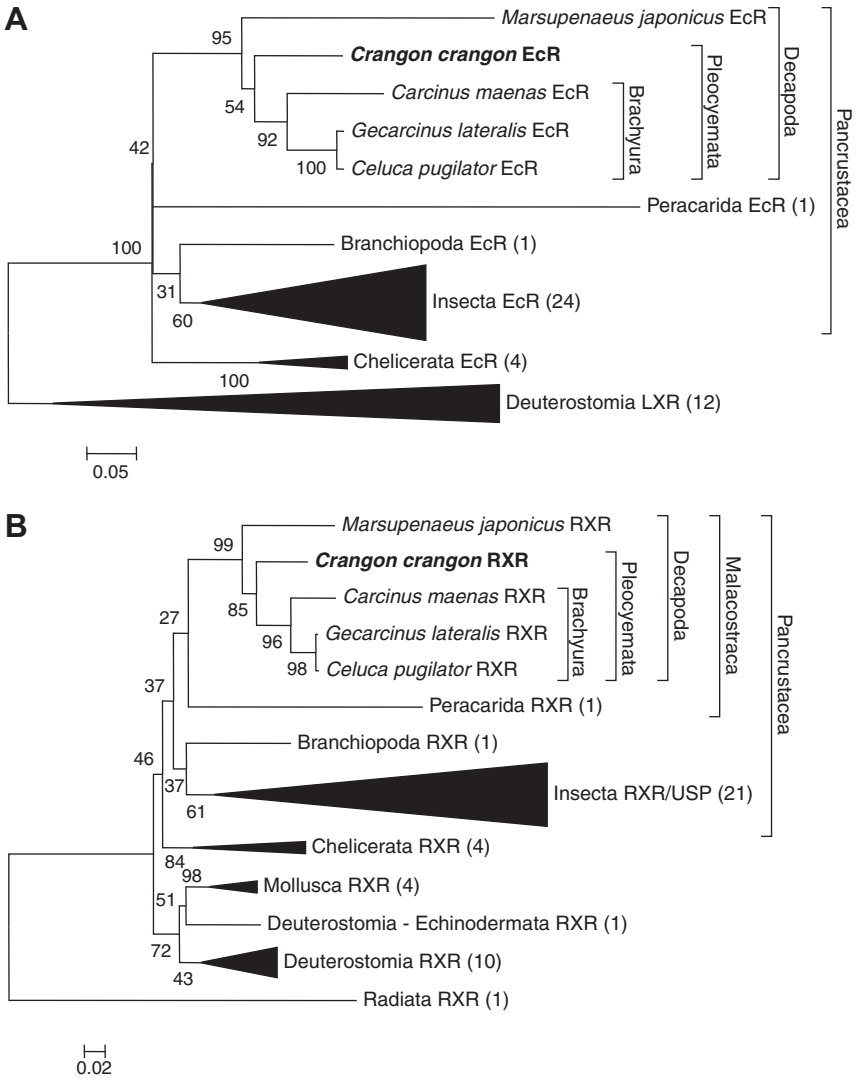

Fig. 2. Phylogenetic trees of the LBD domains of EcR (A) and RXR/USP (B). Trees were constructed using the neighbor-joining algorithm (complete deletion and Poisson correction). The RXR tree was rooted with jellyfish *Tripedalia cystophora*, the EcR tree with the cluster of 11 Deuterostomia LXR(-like) receptors. The figures at the nodes represent bootstrap proportions out of 1000 bootstrap replicates. Branch lengths are proportional to sequence divergence, the bars below each figure represent the number of differences per site. See Supplementary Table S2 in supplementary material for the accession numbers of the used sequences. It should be noted that Hemiptera (*Acyrthosiphon, Bemisia* and *Myzus*), Myriapoda (*Lithobius*), Echinodermata (*Strongylocentrotus*) and the crustacean clade Maxillopoda (*Calanus*) were deliberately excluded from the RXR phylogenetic analysis to promote tree reliability; otherwise Maxillopoda and Hemiptera would cluster with the Hexapoda clade; Myriapoda with Branchiopoda and Chelicerata; and Echinodermata with Mollusca, rendering overall poor bootstrap values (data not shown).

because of quantitative differences in protein interaction with either USP or the transcriptional apparatus in the *Drosophila* host cell line.

The availability of the *Crangon* EcR *in silico* model and the reporter assay based on CrcEcR/RXR expression plasmid transfected Kc L57-3-11 cells can have practical applications to predict endocrine disrupting activities of exogenous compounds. *In silico* generated 3D receptor models are interesting templates for 3D quantitative structure–activity relationship studies (3D QSAR), where correlations between the binding affinity of the ligand molecules and structural features of the common target (i.e. in our case the receptor LBD) are sought. This approach is popular for predicting drug toxicity in vertebrate receptors (Vedani et al., 2006), more recently for vertebrate endocrine disruption (Lill et al., 2005; Iguchi and Katsu, 2008; Cui et al., 2009) or to a lesser extent for insecticide activity in target insect species (Hormann et al., 2008; Nakagawa et al., 2009). However, no such model has been used to our knowledge to investigate the endocrine disrupting potential of drugs and chemicals to important non-target invertebrate species such as *C. crangon*. The described accurate model of the LBD of the shrimp EcR should allow predictive *in silico* docking of potential ligands, thus establishing a shrimp specific high throughput virtual screening system to identify candidate endocrine disrupting compounds (EDCs). Subsequently, the transfected L57-3-11 cell line can be used as *in vitro* bioassay to confirm candidate EDC activity in crustaceans. While a crustacean cell line would be ideal for a cell based bioassay, primary cell cultures obtained from crustaceans are practically unsuited for bioassays due to their limited lifespan. Immortal cell lines require less labor and represent a continuous and reliable bioassay instrument. However, so far no reports on an established crustacean cell line exist (Claydon and Owens, 2008; Lee et al., 2009). To achieve a cell line for investigating the functioning of a crustacean ecdysteroid receptor, non-crustacean cell lines can be transfected with EcR and RXR. Mammalian cell lines have the major advantage that endogenous EcR is naturally absent. Kato et al. (2007) use a complex two-hybrid system with transfected chimeric genes containing the LBDs of *Daphnia magna* EcR and RXR in vertebrate CHO cells. An arthropod cell line is closer to crus-

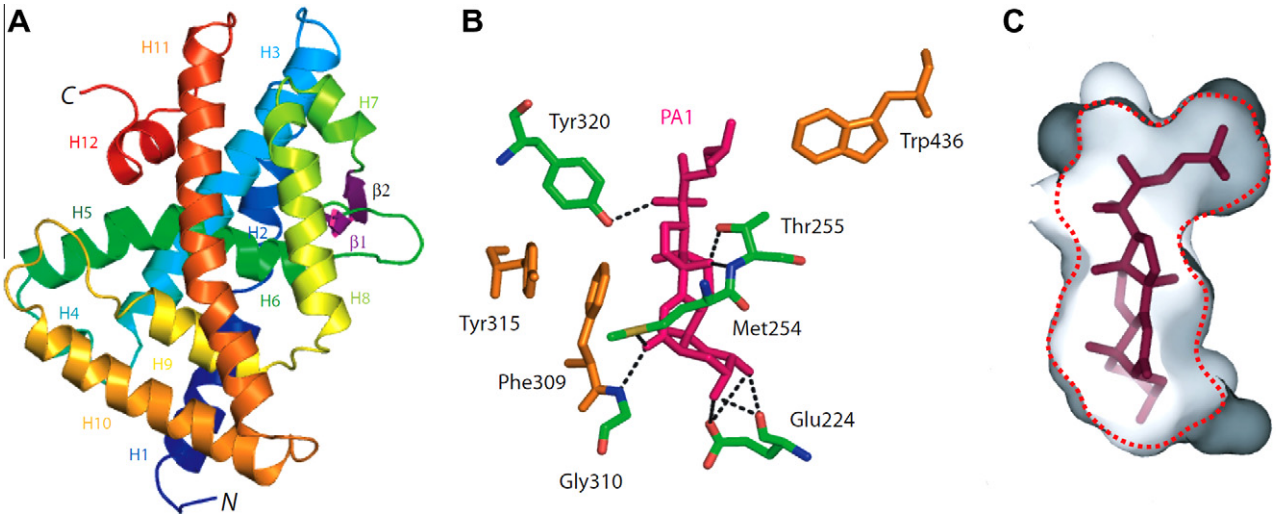

Fig. 3. *In silico* 3D modeling of the EcR ligand-binding pocket. (A) Ribbon diagram of CrcEcR LBD showing the 12 α-helices building the 3D model of the receptor. α-Helices are differently colored and numbered H1-H12, the two short strands of β-sheet are colored purple and numbered β1 and β2. *N* and *C* correspond to the *N*- and *C*-terminus of the polypeptide chain, respectively. (B) PonA complexed to residues forming the ecdysteroid-binding pocket of CrcEcR LBD. PonA is represented in pink stick and H-bonds anchoring PonA to hydrophilic residues are in black dotted lines. Hydrophobic residues making stacking interactions with PonA are colored orange. (C) Clip (red dotted line) showing the orientation of the alkyl chain of PonA in the pocket of the ligand-binding groove of CrcEcR LBD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

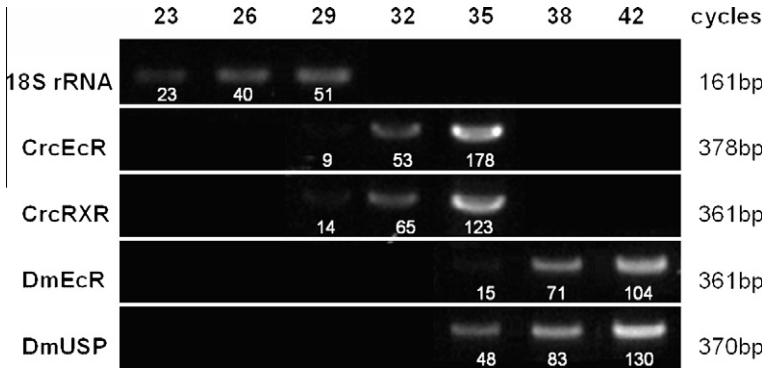

Fig. 4. Quantification of mRNA levels of exogenous CrcEcR/RXR and endogenous DmEcR in transfected L57-3-11 cells by RT-PCR. Intensities (after background subtraction) are given under each band. Fragment lengths are presented at the right in base pairs, and PCR cycle numbers are given above the figure.

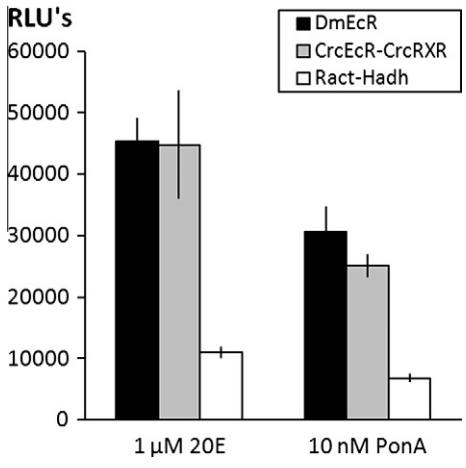

Fig. 5. Functional expression of CrcEcR/RXR in EcR-deficient L57-3-11 cell line. Relative luminescence units (RLUs) are shown after luciferase reporter induction with 1 μM 20E and 10 nM PonA in the L57-3-11 cell line after transfection with CrcEcR-CrcRXR or DmEcR expression plasmids. A third transfection with empty plasmid Ract-Hadh was performed as a negative control. The error bars represent the standard deviation on the average RLUs that is based on four replicate samples.

tacean cells (e.g., posttranslational modifications, co-factors). Based on our results, we believe that using transfected *Drosophila* Kc L57-3-11 cells characterized by a low endogenous EcR expression (Swevers et al., 1996), compounds that influence crustacean molting (Zou, 2005) such as certain pesticides (Schimmel et al., 1979; Baer and Owens, 1999; Snyder and Mulder, 2001; Meng and Zou, 2009; Palma et al., 2009), polychlorinated biphenyls (Fingerman and Fingerman, 1977; Zou and Fingerman, 1999), aromatic hydrocarbons (Cantelmo et al., 1981, 1982), estrogenic agents (Zou and Fingerman, 1997; Montagna and Collins, 2007), phtalates (Zou and Fingerman, 1999) and non-steroidal ecdysteroid agonists used in insect pest control (Weis and Mantel, 1976; Clare et al., 1992; Dhadialla et al., 1998; Waddy et al., 2002; Soin et al., 2010) could be efficiently evaluated for possible endocrine disrupting effects against crustaceans.

An interesting question regarding the functioning of the crustacean ecdysone receptor complex relates to possible interactions between ligands of EcR and those of its heterodimerization partner, RXR. In mammals, ligands of RXR and its heterodimerization partner RAR can have synergistic effects (Roy et al., 1995; Minucci et al., 1997). In mammalian cells transfected with *Daphnia* EcR and RXR Gal4 fusion constructs, activation by 20E is enhanced by

putative RXR ligands (Wang and LeBlanc, 2009). However, structural studies do not support a role for ligand binding in the function of arthropod RXRs (discussed in Tocchini-Valentini et al., 2009). Nevertheless, possible interactions between both nuclear receptors should be taken into account when compounds are screened for endocrine disruption activity.

On a basic research level, the cell line could therefore also present an important tool to compare the functionality and interactions of intra- and interspecies EcR-RXR/USP isoform heterodimers. For example, it would enable to screen potential RXR ligands for their effect on ecdysteroid signaling, which is expected to be different between insects and crustaceans (Fang et al., 2005; Hayward et al., 2003; Iwema et al., 2009; Wang and LeBlanc, 2009). As the consciousness of the important role of RXR in modulating ecdysteroid signaling is increasing, an *in silico* 3D model of CrcRXR LBD would also have its benefits.

5. Conclusion

We report (a) the cloning and characterization of *C. crangon* EcR and RXR, (b) the 3D reconstruction of the EcR LBD and *in silico* docking of PonA, and (c) the functionality of the *C. crangon* ecdysteroid receptor in the mutant *Drosophila* L57-3-11 cell line through an ecdysteroid responsive luciferase reporter construct. Our results suggest that the LBD 3D model and *Drosophila* L57-3-11 cell line may be used to study crustacean ecdysteroid receptors, useful in studying endocrine disrupting effects of chemicals on non-target species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygcen.2010.05.007.

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