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Cloning and characterization of the retinoic acid receptor-like protein in the rock shell, *Thais clavigera*

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ABSTRACT

The organotin compounds have a high affinity for the retinoid X receptor (RXR), which is a transcriptional factor activated by retinoids that induce imposex in gastropods. However, the molecular mechanisms underlying the regulation of RXR and its related genes in gastropods remain unclear. We isolated a retinoic acid receptor (RAR)-like cDNA (*TcRAR*) in the rock shell, *Thais clavigera*, and examined the transcriptional activity of the TcRAR protein by using all-*trans* retinoic acid (ATRA). However, we did not observe any ligand-dependent transactivation by this protein. We also examined the transcriptional activity of the TcRAR-ligand binding domain fused with the GAL4-DNA binding domain by using retinoic acids, retinol, and organotins and again saw no noteworthy transcriptional induction by these chemicals. Use of a mammalian two-hybrid assay to assess the interaction of the TcRAR protein with the TcRXR isoforms suggested that TcRAR might form a heterodimer with the RXR isoforms. The transcriptional activity of domain-swapped TcRAR chimeric proteins (the A/B domain of TcRAR combined with the D–F domain of human RARα) was also examined and found to be ATRA-dependent. These results suggest that TcRAR is not activated by retinoic acids, but can form a heterodimer with TcRXR isoforms. These data contribute to our understanding of the mechanism by which RXR functions in gastropods.

1. Introduction

Pollution by organotin compounds, especially trialkyltins, such as tributyltin (TBT), has spread through aquatic environments worldwide. These polluting compounds have various harmful effects on both vertebrates and invertebrates (Boyer, 1989; Fent, 1996; Golub and Doherty, 2004). A classic effect of these organotins on invertebrates is the induction of imposex in female gastropods; imposex is defined as an irreversible pseudohermaphroditic condition in which male genital organs, such as the penis and vas deferens, develop in female prosobranch gastropods (Bryan et al., 1986; Smith, 1971). Since the mid-1960s, organotins such as TBT and triphenyltin (TPT) have been used worldwide in antifouling paints for ships and fishing nets (Bryan et al., 1986, 1987, 1988; Gibbs et al., 1987; Horiguchi et al., 1994, 1997a), and consequently approximately 200 species of Caenogastropoda, including the rock shell, *Thais clavigera*, have been affected by imposex (Bech, 2002a,b; Fioroni et al., 1991; Horiguchi, 2000; Horiguchi et al., 1997b; Marshall and Rajkumar, 2003; Matthiessen et al., 1999; Shi et al., 2005; Sole et al., 1998; ten Hallers-Tjabbes et al., 2003; Terlizzi et al., 2004). Gastropod imposex can cause reproductive failure (i.e., either oviduct blockage by the formation of vasa deferentia or ovarian spermatogenesis in the later stages of imposex), which could lead to population decline or mass extinction, especially in the gastropod species whose planktonic stages are short (Gibbs and Bryan, 1986; Gibbs et al., 1988, 1990; Horiguchi et al., 2006). Previous studies have reported that TBT and TPT function as ligands that activate the transcription of the retinoid X receptor (RXR) in humans, crustaceans, and gastropods (Nishikawa et al., 2004; Wang and LeBlanc, 2009). In *T. clavigera*, a single injection of either 9-*cis* retinoic acid (9cRA) or TPT into females induces imposex development, suggesting that imposex is induced through the activation of RXR (Nishikawa et al., 2004). These observations suggest that retinoic acid receptors could be involved in the induction of imposex development in prosobranch gastropods.

Retinoic acids are signaling molecules with regulatory effects on pattern formation, cell differentiation, embryonic development, and maintenance of numerous tissues in vertebrates (see review in

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Maden and Hind, 2003; Mark et al., 1999, 2006; Niederreither and Dollé, 2008; Samarut and Rochette-Egly, 2012). In the absence of vitamin A during rat development, a broad array of malformations is observed: ocular defects (e.g., post-lenticular fibroplasia), cardiac anomalies (e.g., interventricular septal defect), respiratory defects (e.g., agenesis and rudimentary lung), and genitourinary tract anomalies (e.g., absence/incomplete development of the homologous set of Müllerian or mesonephric ducts, cryptorchidism) (Wilson et al., 1953). Vitamin A (retinol), a principal circulating form of retinoid, and its derivative retinoids, are converted into all-*trans* retinoic acid (ATRA) and 9cRA (Albalat, 2009; Chen et al., 2000; see review in Marill et al., 2003; Nagao, 2004; Napoli, 1996). The retinoids activate gene transcription via two families of retinoic acid receptors: retinoic acid receptors (RARs) and RXRs (Blumberg et al., 1997; Chambon, 1996). ATRA has high affinity only for RARs, whereas 9cRA binds with high affinity to both RARs and RXRs.

There are three RAR isoforms (α, β, and γ) and three RXR isoforms (α, β, and γ), and they have ligand-dependent transactivation functions in vertebrates (Bastien and Rochette-Egly, 2004; Chambon, 1996). The RXRs form heterodimers with RARs and various nuclear receptors, such as vitamin D receptor, thyroid hormone receptor, peroxisome proliferator-activated receptor, liver-X receptor, bile acid receptor, and xenobiotic compound receptor (Wolf, 2006). The cellular mechanisms controlled by the RXR/RAR heterodimers are well defined. The heterodimers bind to direct repeats (DR) of a specific DNA sequence, known as the retinoic acid response element (RARE); the DRs are spaced 1–5 nucleotides apart (DR1 to DR5). RAR/RXR heterodimers can also bind to a DR5-type element (Mangelsdorf and Evans, 1995) or several RAREs (DR1 type, DR2, and IR-0) (Balmer and Blomhoff, 2005; La Vista-Picard et al., 1996; Mangelsdorf and Evans, 1995). These RAREs also contain a core motif, (A/G)G(G/T)TCA (Mangelsdorf and Evans, 1995; Samarut and Rochette-Egly, 2012).

In vertebrates, RAR plays important roles in the formation of reproductive organs. In *RARα*-null mutant mice, testis-degeneration was observed (Lufkin et al., 1993). Moreover, synergistic transcriptional activity and binding in the presence of both human RARα and RXRα have been observed in vitro (Husmann et al., 1992). In an in vivo study, *RARs/RXRα* double mutant mice showed partial or complete agenesis of the Müllerian duct (Kastner et al., 1997). These results suggest that RXR/RAR heterodimers are required for efficient physiological function in vertebrates.

Among invertebrates, several different RXR isoforms have been reported in gastropods (Bouton et al., 2005; Castro et al., 2007; Nishikawa et al., 2004; Urushitani et al., 2011). Candidate partners for RXR heterodimerization (e.g., RAR or RAR-like protein) have been reported for the ascidian *Polyandrocarpa misakiensis* (Hisata et al., 1998), the mollusc (limpet) *Lottia gigantea* (Albalat and Cañestro, 2009), and the crustacean *Daphnia pulex* (Thomson et al., 2009). In ascidians, retinoic acid induces ectopic gut differentiation (Hara et al., 1992). In addition, ascidian RAR binds the DR-5 element in the presence of ascidian RXR (Kamimura et al., 2000). In molluscs, genomic analysis has revealed the presence of RAR and retinoic acid related metabolic enzyme (Aldh1a and Cyp26)-like genes (Albalat, 2009; Albalat and Cañestro, 2009). However, it is unclear whether these receptor- and enzyme-like proteins have functional roles in retinoic acid cascades. Moreover, the dimerization capability and functions of molluscan RAR-like protein and RXRs are also still unknown.

In our previous studies on isoforms/variants of RXRs, we obtained two cDNA clones encoding RXRs (RXR isoform 1 [*TcRXR-1*] and RXR isoform 2 [*TcRXR-2*]) and performed transactivation assays with them. The TcRXR-1 displayed 9cRA, TBT, and TPT dose-dependent activation of transcription (Urushitani et al., 2011). Our findings from these studies suggested that RXR might be activated by retinoic acid and induce the development of male-type genital tracts (penis and vas deferens) in normal male and organotin-exposed female rock shells. These findings also suggested that heterodimer partners for RXR are likely present in the rock shell and that functional analysis for a candidate partner for the rock shell RXR should be conducted to better understand the mechanism by which imposex is induced in prosobranch gastropods.

Accordingly, we attempted to isolate the RAR cDNA in rock shell. We then performed a functional analysis of this RAR-like (TcRAR) protein by using a reporter gene assay that included retinoids. Finally, to determine whether TcRXR isoforms and TcRAR form functional heterodimers, we performed a mammalian two-hybrid assay. We found a rock shell RAR-like protein that was not activated by retinoic acids, but heterodimerized with RXR isoforms. Thus, this paper is the first report of the functional analysis of a molluscan RAR-like protein.

2. Materials and methods

2.1. Animals and chemical reagents

Male rock shells (*T. clavigera*) were collected in October 2010 at Hiraiso, Ibaraki Prefecture, Japan, an area known to have minimal organotin contamination (less than 10 ng/g wet wt and 8 ng/g wet wt for TBT and TPT, respectively; see Horiguchi et al., 1997a). The snails were dissected immediately after collection and preserved in RNAlater solution (Life Technologies Corporation, Carlsbad, CA, USA) for RNA extraction. These samples were stored at −80 °C until use.

9cRA, ATRA, 13-*cis* retinoic acid (13cRA), *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA), tributyltin chloride (TBTCl), and triphenyltin chloride (TPTCl) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All-*trans* retinol (AtRetinol) was purchased from LKT Laboratories, Inc (St. Paul, MN, USA). All chemicals were dissolved in dimethylsulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan), the concentration of which in the culture medium did not exceed 0.1%.

2.2. Cloning of T. clavigera RAR (TcRAR)

To identify *TcRAR*, degenerate oligonucleotide primers were designed based on the DNA-binding domain (DBD) (Fw: KNCINKV) and the ligand-binding domain (LBD) (Rv: KMLMKITD) of RAR in vertebrates and invertebrates (Table 1). Total RNA was isolated from snails using Isogen (NIPPON GENE, Tokyo, Japan) and an RNeasy kit (Qiagen, Venlo, Netherlands) that included a DNase-I treatment. RNA quantity and quality were checked by measuring their optical density and by gel electrophoresis. First-strand cDNA was synthesized from 2 μg of total RNA isolated from the penis or kidney of *T. clavigera*. Then, partial fragments of target cDNAs were amplified by polymerase chain reaction (PCR) with AmpliTaq Gold® DNA polymerase (Life Technologies) using oligonucleotides under suitable conditions (Table 1). The amplified DNA fragments were subcloned into the vector pCR2.1 (Life Technologies) and sequenced by using a BigDye Terminator Cycle Sequencing-kit (Life Technologies) and the ABI PRISM 3730 sequencer (Life Technologies).

The 5′- and 3′-ends of the *TcRAR* were amplified by rapid amplification of the cDNA end (RACE) with a SMART RACE cDNA amplification kit (Clontech Laboratories Inc., Mountain View, CA, USA). The 5′- and 3′-RACE were performed with gene-specific primers (Table 2) according to manufacturer's instructions. The full-length transcript encoding the open reading frame was then PCR amplified with KOD Plus DNA polymerase (Toyobo Biochemicals, Osaka, Japan) using a primer pair specific for the 5′- and 3′-untranslated regions (Table 1). The resultant amplification products were subcloned into pCR-Blunt II (Life Technologies) and their sequences were confirmed by sequence analysis.