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Cloning and characterization of retinoid X receptor (RXR) isoforms in the rock shell, *Thais clavigera*

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ABSTRACT

The organotin compounds tributyltin (TBT) and triphenyltin (TPT) belong to a diverse group of widely distributed environmental pollutants that induce imposex in gastropods. These organotins have high affinity for retinoid X receptor (RXR), which is a transcription factor activated by retinoids, such as 9-*cis* retinoic acid (9cRA), in vertebrates. However, the molecular mechanisms underlying the regulation of RXR by retinoids and organotins have not been clarified in gastropods. We isolated two isoforms of RXR cDNAs, RXR isoform 1 (*TcRXR-1*) and RXR isoform 2 (*TcRXR-2*), in the rock shell *Thais clavigera*. The deduced amino acid sequences of *TcRXR-1* and *TcRXR-2* are highly homologous with those of other gastropods. These TcRXR isoforms displayed 9cRA-dependent activation of transcription in a reporter gene assay using COS-1 cells. The transcriptional activity of TcRXR-2, the encoded protein of which has five additional amino acids in the T-box of the C domain, was significantly lower than that of TcRXR-1. Decreases of the transcriptional activity by TcRXR-1 were observed when more than equal amount of *TcRXR-2* fused expression vector was existed in a co-transfection assay. Immunoblot analysis showed several shifted bands for TcRXR isoforms resulting from phosphorylation. Mutation of potential phosphorylation sites from serine to alanine in the A/B domain of TcRXR-1 showed that, in the S89A/S103A mutant, there was a band shift and significantly higher transcriptional activity than in the controls when stimulated with 9cRA. Our findings could contribute to a better understanding of the role of interactions between RXR and retinoids and organotins, not only in the induction mechanism of imposex in gastropods but also in the endocrinology of mollusks.

1. Introduction

Retinoic acids (RAs) play important roles in cell proliferation, differentiation, and development in vertebrates (Ross et al., 2000). Retinol, a principal circulating form of retinoid, is converted into all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9cRA) (Albalat, 2009; Chen et al., 2000; Nagao, 2004; Napoli, 1996). These retinoids have pleiotropic effects through two classes of nuclear receptor, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Blumberg et al., 1997; Chambon, 1996). These receptors have ligand-dependent transactivation functions. RARs are activated by ATRA and 9cRA heterodimerized with RXRs, whereas RXRs are activated only by 9cRA (Bastien and Rochette-Egly, 2004). In vertebrates, there are three RAR isoforms (α, β, γ) and three RXR isoforms (α, β, γ). RXRs form heterodimers with nuclear receptors, vitamin D receptor, thyroid hormone receptor, peroxisome proliferator-activated receptor, liver-X receptor, bile acid receptor, and xenobiotic compound receptor (Wolf, 2006). RXR/RAR heterodimers bind to direct repeats (DR) of a specific DNA sequence, known as the retinoic acid response element (RARE), that are spaced by 1–5 nucleotides (DR1–DR5). RXR homodimers can also bind to a DR1-type element in the retinoid X response element (Mangelsdorf and Evans, 1995). Among the invertebrates, the ecdysteroid receptor (EcR) forms heterodimers with RXR in crustaceans (Durica et al., 2002; Hopkins et al., 2008). Candidate partners for RXR heterodimerization (e.g., RAR or RAR-like protein) have been reported in the ascidian *Polyandrocarpa misakiensis* (Hisata et al., 1998), the mollusk *Lottia gigantea* (Albalat and Cañestro, 2009), and the crustacean, *Daphnia pulex* (Thomson et al., 2009); however, it is unknown whether receptors other than EcR dimerize with RXR. Several different RXR isoforms have been reported in gastropods (Bouton

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et al., 2005; Castro et al., 2007) and crustaceans (Asazuma et al., 2007; Durica et al., 2002; Kim et al., 2005; Priya et al., 2009), and one cDNA clone of RXR has been reported in the rock shell, *Thais clavigera* (Nishikawa et al., 2004). These findings suggest that isoforms or variants of RXR could also be present in the rock shell.

Nuclear hormone receptors exhibit a conserved modular structure consisting of six regions (A–F). The N-terminal A/B region of these receptors is highly variable in sequence and length (Rochette-Egly, 2003) and contains an autonomous ligand-independent transcriptional activation domain called AF-1. The AF-1 domain contains multiple consensus phosphorylation sites (Rochette-Egly, 2003). In mammals, RXRα can be phosphorylated at several serine and threonine residues within the A/B domain (Adam-Stitah et al., 1999). Phosphorylation of RXRα is required for the activation of a subset of RA target genes and for the anti-proliferative effect of RA, as shown by using the rescue strategy in F9 cells (Bastien et al., 2002). Moreover, the N-terminal domain in the RXRα-deleted mutant mouse is thought to have an important function in the cascade of molecular events that lead to the normal disappearance of interdigital mesenchyme by A/B domain phosphorylation (Mascrez et al., 2001).

Binding of specific ligands to RXRs activates the ability of receptors to regulate the transcription of responsive genes. *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA) binds and activates RXR in vertebrates (de Urquiza et al., 2000). In humans, crustaceans, and gastropods, organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) have been reported as ligands that activate the transcription of RXR (Nishikawa et al., 2004; Wang and LeBlanc, 2009). One of the most toxic effects of TBT and TPT in gastropods is the induction of imposex, 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). Imposex is typically induced by TBT or TPT, or both, at very low concentrations (~1 ng/l); these compounds have been used in antifouling paints for ships and fishing nets since the mid-1960s (Bryan et al., 1986, 1987, 1988; Gibbs et al., 1987; Horiguchi et al., 1994, 1997a). Reproductive failure in gastropods can occur in the later stages of imposex, either because of oviduct blockage by the formation of vasa deferentia or because of ovarian spermatogenesis. This failure eventually results in population decline or mass extinction (Gibbs and Bryan, 1986; Gibbs et al., 1988, 1990; Horiguchi et al., 2006). Approximately 200 species of mesogastropods and neogastropods, including the rock shell, *T. clavigera*, are 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). Imposex among gastropods is clear evidence of endocrine disruption caused by environmental pollutants (Matthiessen and Gibbs, 1998; Matthiessen et al., 1999). Six hypotheses have been proposed to explain the mechanisms by which TBT induces imposex in gastropods: (1) an increase in androgen (e.g., testosterone) levels as a result of TBT-mediated inhibition of aromatase (Bettin et al., 1996); (2) an increase in testosterone levels owing to the inhibition of acyl CoA-steroid acyltransferase (Gooding et al., 2003; Sternberg and LeBlanc, 2006); (3) TBT-mediated inhibition of the excretion of androgen sulfate conjugates, with a consequent increase in androgen levels (Ronis and Mason, 1996); (4) TBT interference with the release of penis morphogenetic/retrogressive factor from the pedal/cerebropleural ganglia (Féral and Le Gall, 1983); (5) an increase in the level of an alanine-proline-glycine-tryptophan amide neuropeptide in response to TBT (Oberdörster and McClellan-Green, 2000); and (6) activation of RXR (Nishikawa et al., 2004).

Nishikawa et al. (2004) demonstrated that the rock shell (*T. clavigera*) RXR (TcRXR) binds both 9cRA and organotins and that a single *in vivo* injection of 9cRA into female rock shells with no morphological signs of imposex induces the development of imposex a month later, although transcriptional activity of TcRXR has not been tested. Our previous studies of RXR gene expression and RXR protein content were performed in various tissues of male and female wild *T. clavigera* by using quantitative real-time reverse transcription PCR, Western blot analysis, immunohistochemistry with a specific antibody against TcRXR, injection of female *T. clavigera* with 9cRA at three different concentrations, and time-course analysis of RXR gene expression and induction of imposex in female *T. clavigera* exposed to TPT for 3 months (Horiguchi et al., 2007, 2008a, 2010a,b). Taken together, our findings from these studies suggested that RXR might be involved in inducing the development of male-type genitalia and their components (penis and vas deferens) in normal male and organotin-exposed female rock shells.

Here, to investigate the presence of RXR isoforms/variants, we isolated two cDNA clones encoding RXR – RXR isoform 1 (*TcRXR-1*) and RXR isoform 2 (*TcRXR-2*) – and performed transactivation assays in COS-1 cells. Transcriptional activity of TcRXR-2 was lower than that of TcRXR-1 in the RXRE reporter gene assay, and decreases of the transcriptional activity of TcRXR-1 were observed when co-transfections were performed with more than equal amount of *TcRXR-2* fused expression vector. TcRXR-1 and TcRXR-2 were phosphorylated in COS-1 cells, and we identified at least two phosphorylated serine residues in the A/B domain of TcRXR-1 by analysis of point mutations. These data could contribute to our understanding of the fundamental mechanism by which RXR functions in gastropods.

2. Materials and methods

2.1. Animals and chemical reagents

Male rock shells (*T. clavigera*) were collected during October 2008 at Hiraiso, Ibaraki Prefecture, Japan, an area known to have minimal organotin contamination (less than 10 ng/g wet wt. for tributyltin chloride (TBTCl) or less than 8 ng/g wet wt. for triphenyltin chloride (TPTCl); see Horiguchi et al., 1997a). The snails were dissected immediately after collection and preserved in RNA*later* solution (Applied Biosystems, Foster City, CA, USA) for RNA extraction, or were treated by liquid nitrogen for protein extraction. These samples were stored at −80 °C until use.

9cRA, DHA, TBTCl, and TPTCl were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All chemicals were dissolved in dimethylsulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan). The concentration of DMSO in the culture medium did not exceed 0.1%.

2.2. Cloning of T. clavigera RXRs (TcRXRs)

To identify *TcRXR* subtypes, degenerate oligonucleotide primers were designed based on the DNA-binding domain (DBD) (Fw: CEGCKGFF, Rv: CQYCRLQKC) of RAR and RXR in vertebrates and invertebrates, and then performed RT-PCR. First-strand cDNA was synthesized from 2 μg total RNA isolated from the penis of *T. clavigera*. The amplified DNA fragments were subcloned into the vector pCR2.1 (Invitrogen, Carlasbad, CA, USA) and sequenced by using a BigDye Terminator Cycle Sequencing-kit (Applied Biosystems) and the ABI PRISM 377 sequencer (Applied Biosystems).

The 5′- and 3′-ends of the *TcRXR*s were amplified by rapid amplification of the cDNA end (RACE) with a SMART RACE cDNA amplification kit (Clontech Laboratories Inc., Mountain View, CA, USA). Full-length transcript encoding the open reading frame was then PCR amplified with KOD Plus DNA poly-