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Development of yeast reporter assays for the enhanced detection of environmental ligands of thyroid hormone receptors α and β from *Xenopus tropicalis*

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ABSTRACT

Thyroid hormones (THs) are involved in the regulation of metabolic homeostasis during the development and differentiation of vertebrates, particularly amphibian metamorphosis, which is entirely controlled by internal TH levels. Some artificial chemicals have been shown to exhibit TH-disrupting activities. In order to detect TH disruptors for amphibians, we herein developed a reporter assay using yeast strains expressing the thyroid hormone receptors (TRs) α and β together with the transcriptional coactivator SRC-1, all of which were derived from the frog *Xenopus tropicalis* (XT). These yeast strains responded to endogenous THs (T_2, T_3, and T_4) in a dose-dependent manner. They detected the TR ligand activities of some artificial chemicals suspected to exhibit TH-disrupting activities, as well as TR ligand activity in river water collected downstream of sewage plant discharges, which may have originated from human excrement. Moreover, the responses of XT TR strains to these endogenous and artificial ligands were stronger than those of yeast strains for human TRα and β assays, which had previously been established in our laboratory. These results indicate that the yeast reporter assay system for XT TRα and β is valuable for assessing TR ligand activities in environmental samples that may be particularly potent in amphibians.

1. Introduction

Thyroid hormones (THs) are essential endocrine signaling molecules that control metabolic homeostasis during the development and differentiation of vertebrates (Yen, 2001; Warner and Mittag, 2012; Kulkarni and Buchholz, 2013). Two THs, 3,5,3′,5′-tetraiodothyronine (thyroxine, T_4) and 3,5,3′-triiodothyronine (T_3) are synthesized in the thyroid gland under the control of thyroid-stimulating hormone. T_4 is primarily secreted into the blood, and is converted to the active form T_3 by deiodinases in cells. T_4 is also metabolized into other inactive iodothyronine products, such as 3,3′,5′-triiodothyronine (reverse T_3), 3,3′-diiodothyronine (T_2), 3′-monoiodothyronine (T_1), and thyronine (T_0). THs are metabolized to water-soluble conjugated forms by sulfation or the formation of glucuronides in the liver, and are excreted by the kidney or in bile. This process contributes to the conservation of body iodine stores (Kogai and Brent, 2005; Morvan-Dubois et al., 2008).

TH signaling requires thyroid hormone receptors (TRs), which are members of the nuclear receptor superfamily. TRs are transcription factors that regulate gene expression by binding to DNA at specific sites qualified as thyroid hormone response elements (TRE). TREs are generally composed of two half sites with the consensus motif sequence 5′-AGGTCA-3′ (Brent et al., 1989). A representative TRE with a direct repeat of the half sites separated by 4 bp is referred to as the DR-4 element (Suen et al., 1994). Other types of TREs are IR-0 (an inverted repeat with no spacer between the half sites) and ER-6 (an everted repeat with a 6-bp spacer) (Glass et al., 1988). Liganded TRs recruit coactivator complexes on TRE in order to facilitate transcription. In contrast, in the absence of T_3, TRs repress transcription by recruiting corepressor complexes (Damm et al., 1989; Paul et al., 2007). Two TR genes, *TRα* and *TRβ*, are conserved in vertebrates (Lazar, 1993), and the relative expression of these two TR genes and the distribution of their products vary among tissues and during different stages of development (Hodin et al., 1990; Yaoita and Brown, 1990). Moreover, cross-regulation has been demonstrated between the TR isoforms. The regulation of TRβ expression in the liver and heart is known to be controlled by TRα (Sadow et al., 2003).

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Amphibian metamorphosis shares a number of similarities with postembryonic development in mammals (Tata, 1998). Amphibian metamorphosis is divided into three developmental periods: premetamorphosis, prometamorphosis, and climax (Dodd and Dodd, 1976). Premetamorphosis is the truly larval period, during which the tadpole grows and develops under the control of maternal T_3, prior to the formation of a functional thyroid gland. In prometamorphosis, the most prominent change is the development of both sets of limbs, and morphological alterations to the adult frog are almost complete by climax. In climax, the cloacal tailpiece begins to shrink, and upon tail resorption, all radical changes end. These metamorphic processes are entirely controlled by T_3 and TRs. In premetamorphosis, TRα mRNA levels increase and peak by prometamorphosis. TRβ mRNA is barely detectable during premetamorphosis. In prometamorphosis, endogenous T_3 is synthesized by the thyroid gland, and TRβ mRNA is up-regulated by endogenous T_3. Endogenous T_3 and TRβ mRNA levels peak at climax, and then rapidly decrease to a minimum in parallel with TRα mRNA levels (Yaoita and Brown, 1990).

Population decreases, reproductive anomalies, and malformations have been increasingly reported in highly aquatic animals since the 1990s (Kingsford et al., 1996; Houlahan et al., 2000; Vandenlangenberg et al., 2003). One possible cause of these events is considered to be the endocrine disruptive effects induced by environmental contaminants through direct actions on nuclear receptors (Burkhart et al., 1998; Vos et al., 2000). Polychlorinated biphenyls (PCBs), which are industrial chemicals used as coolants and lubricants in electrical equipment, have been suggested to interact with TR because of their structural similarities to THs (McKinney and Waller, 1994). Bisphenol A (BPA), which is used to produce polycarbonate plastic, has been shown to bind to TR as an antagonist (Moriyama et al., 2002). The detergent component nonylphenol has been reported to weakly interfere with TH binding to the TH transport protein transthyretin (TTR) (Ishihara et al., 2003). Chlorinated derivatives of BPA and NP have been shown to inhibit TH binding not only to TTR, but also to the ligand binding domain (LBD) of chicken and bullfrog TR (Yamauchi et al., 2003). *o*-*t*-Butylphenol (OBP), 2-isopropylphenol (IPP), tetrabromobisphenol A (TBBPA), and tetramethylbisphenol A (TMBPA), other industrially produced substances, also exhibit TH-disrupting activities (Kitamura et al., 2005; Kudo and Yamauchi, 2005; Shiizaki et al., 2010).

We have been attempting to establish yeast reporter assay systems to screen nuclear receptor ligands (Kawanishi et al., 2003, 2006; Chu et al., 2009a, 2009b; Shiizaki et al., 2010, 2014; Ito-Harashima et al., 2015), and they have been applied in various studies on environmental contaminants (Kawanishi et al., 2004; Chu et al., 2009b). These bioassays are based on the quantification of reporter enzymes that reflect ligand-dependent nuclear receptor activation in yeast cells. They are simpler, easier to handle, and less expensive than mammalian cell-based bioassays. Moreover, since yeasts do not express endogenous nuclear receptor homologs (Goffeau et al., 1997), they may be used to detect ligands that are specific to individual receptor subtypes.

In the present study, we developed reporter assay systems using yeast strains expressing TRα and TRβ along with the transcriptional coactivator SRC-1, all of which were derived from the frog *Xenopus tropicalis* (XT). Since amphibian metamorphosis is entirely controlled by TRs (reviewed by Brown and Cai, 2007), a yeast system that mimics TR-mediated gene signaling in the amphibian is considered advantageous for evaluating the ecotoxicological potencies of environmental samples. We examined the responses of these frog TR strains to endogenous THs, chemicals suspected to be TR ligands, and several environmental samples. Furthermore, we compared the ligand responses of XT TR strains with those of our human TR strains that had been established previously (Shiizaki et al., 2010).

2. Materials and methods

2.1. Chemicals

Dimethyl sulfoxide (DMSO), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), T_3, and TMBPA were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). 3,5-Diiodo-L-thyronine (T_2) and L-thyroxine (T_4) were obtained from MP Biomedicals, LLC (Santa Ana, *CA*, USA). TBBPA, IPP, and BPA were obtained from Tokyo-Kasei Co., Ltd. (Tokyo, Japan). OBP and dithiothreitol (DTT) were purchased from Nacalai Tesque (Kyoto, Japan). *p*-*n*-Nonylphenol (NP) was purchased from WAKO Pure Chemicals (Osaka, Japan).

2.2. Cloning of XT TRα, TRβ, and SRC-1 cDNAs

XT frogs were provided by the National BioResource Project (NBRP) of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), XenoBiores at Hiroshima University, Japan. Total RNA was extracted from the livers of adult frogs using ISOGEN (Nippon Gene, Tokyo, Japan). The DNA fragments containing the open reading frames (ORFs) of XT TRα (NM_001045796), TRβ (NM_001045805), and SRC-1 (NM_001112912) were obtained by a reverse-transcription polymerase chain reaction (RT-PCR). The synthesis of cDNA was performed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, *CA*) with 1.8 μg of total RNA as the template. The ORFs of these genes were amplified by PCR using KOD-Plus (TOYOBO Co., Ltd., Osaka, Japan) from cDNA with the primer pairs shown in Table 1. Primers containing a restriction site and/or yeast ribosomal binding consensus sequence near the initiation codon were designed based on the nucleotide sequences registered in the DDBJ/EMBL/GenBank database. Regarding XT TRα cDNA, nested PCR was performed using xTRα1F and xTRα1R in first-step PCR, and xTRα2F and xTRα2R in second-step PCR. The primer pairs xTRβF and xTRβR, and xSRC1F and xSRC1R were used to amplify XT TRβ and SRC-1 cDNA, respectively.

2.3. Plasmids

Plasmids expressing nuclear receptors and coactivators were constructed for XT TRα and TRβ ligand assays. Detailed maps of the plasmids are shown in Fig. 1. The nuclear receptor expression plasmids pUdp6-xTRα and pUdp6-xTRβ (Fig. 1A) were generated by inserting XT TRα and TRβ cDNAs, respectively, into the *Xma*I-*Eco*RI sites of multiple cloning site 2 (MCS2) of the pUdp6 vector without a yeast replication origin, which had previously been constructed in our laboratory (Shiizaki et al., 2010). In order to construct the coactivator expression plasmid pESC-Leu-xSRC1, XT SRC-1 cDNA was cloned into the *Pac*I-*Not*I sites of MCS1 of the pESC-leu vector (Stratagene, La Jolla, USA) (Fig. 1B). The expression of these genes was under the control of the

Table 1
Oligonucleotides used in the study for nuclear receptor and coactivator cDNA amplification.

xTRα1F	ATGAGCCTCGGGCAGCTGGG
xTRα1R	ATGGCCAACCAAGGCAGGGA
xTRα2F[a,b]	CCCCCCCGGG*AACAAA*ATGGACCAGAATCTCAGCGGGCT
xTRα2R[a]	CCCCGAATTCTCAGACTTCCTGGTCCTCAAAGA
xTRβF[a,b]	CCCCCCCGGG*AACAAA*ATGCCAAGCAGTATGTCAGAGAC
xTRβR[a]	CCCCGAATTCCTAGTCCTCAAACACTTCCAAGA
xTRβfPCRF[c]	TCCCATAGTTAGTGCGCCTGAGG
xTRβfPCRR[c]	CCTCAGGCGCACTAACTATGGGA
xSRC1F[a,b]	CCCCGCGGCCGC*AACAAA*ATGAGTGGCCTCGGGGACAGTT
xSRC1R[a]	CCCCTTAATTAATCATTCCGTCAGCAGTTGCTGT

[a] Underlined sequences are additional nucleotides to introduce restriction enzyme recognition sites.
[b] The sequences shown in italics are yeast ribosomal binding consensus sequences.
[c] Underlined sequences are a mutation to create an Asn^{179} to Ser^{179} substitution in XT TRβ.