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Establishment of yeast reporter assay systems to detect ligands of thyroid hormone receptors α and β

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ABSTRACT

Thyroid hormones are essential for proper development and differentiation in vertebrates. Recently, concern over the disruption of thyroid hormone homeostasis by industrial chemicals and environmental pollutants has been spreading. To evaluate these chemicals, several bioassays have been developed to detect thyroid hormone ligand activity. Nevertheless, a simple and useful assay is required for the assessment of an enormous number of environmental chemicals. We established yeast reporter assays by expression of full-length thyroid hormone receptor (TRα or TRβ) cDNA and of the TR-dependent reporter gene in yeasts. By additional introduction of the general coactivator SRC-1 cDNA into the yeasts, a higher response to endogenous thyroid hormones, thyroxine (T4), and triiodothyronine (T3) was obtained. The EC50 values for T3 were 35 and 1.5 nM for TRα and TRβ assay yeasts, respectively. We tested four chemicals, tetrabromobisphenol A, tetramethylbisphenol A, 2-isopropylphenol, and *o-t*-butylphenol, which are suspected to have thyroid hormone-disrupting activity. All four chemicals showed agonistic activities in both assay yeasts; however, their activities were weak in comparison with endogenous TR ligands. Antagonist activities of 2-isopropylphenol and *o-t*-butylphenol were also found in the TRα yeast assay. Taken together, these assay yeasts will be powerful tools for assessing TR ligand activity of industrial chemicals and environmental pollutants.

1. Introduction

The thyroid hormones (THs) are widely distributed in a range of vertebrates and are essential for proper development and differentiation. These hormones, thyroxine (T4) and triiodothyronine (T3), are tyrosine-based hormones produced by the thyroid gland. The thyroid hormones regulate energetic homeostasis by increasing the basal metabolic rate affecting protein synthesis and carbohydrate metabolism (Yen, 2001). T4 is the predominant form of THs in the blood and is converted to T3 by deiodinases in the cells. Most biological activities of THs are due to T3 because it has a 10–15-fold higher affinity for binding to thyroid hormone receptors (TRs) than T4 (Togashi et al., 2005). 3,5-Diiodo-L-thyronine (T2) found *in vivo* as a T3 metabolite bound to TR with a very low affinity. Interestingly, T2 can repress TSH gene expression in a similar dose to T3 *in vivo*, so T2 may be involved in the non-TRs pathway (Moreno et al., 2008).

The biological actions of THs are mediated by TRs that are members of a large family of nuclear receptors including steroid hormone receptors (Evans, 1988). The ligand-bound TRs heterodimerize with retinoid X receptor α (RXRα) and can bind to TH response elements (TREs) (Lazar et al., 1990). The TR/RXRα heterodimer causes transcriptional activation of downstream genes of TREs. In addition, transcriptional activation requires recruitment of coactivators such as steroid receptor coactivator-1 (SRC-1) to the TR/RXRα complex (Takeshita et al., 1996). TREs consist of two copies of the consensus motif sequence 5′-AGGTCA-3′ (Brent et al., 1989). A representative TRE that has two copies of the motif arranged as a direct repeat separated by 4 bp is referred to as a DR-4 element (Suen et al., 1994). Another TRE with everted repeats (ER-6) is also common. A third TRE with head-to-head repeats without base pair separation, inverted repeat (IR-0), is less common (Glass et al., 1988). There are two major TR isoforms encoded by different genes, which are designated as TRα and TRβ (Sap et al., 1986; Weinberger et al., 1986; Jansson et al., 1983; Thompson et al., 1987). In mammalian species, TRα and TRβ contain a highly homologous DNA-binding domain (DBD) and a ligand-binding domain (86% and 82%, respectively, in amino acid sequences) (Lazar et al., 1990). Both TR isoforms bind to T3 and mediate TH-regulated gene expression, but their physiological functions are not the same. Mice with null mutant or dominant negative mutant of each receptor display different phenotypes. The inactivation of the TRβ gene leads to hyperthyroxinemia by

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the disorder of the pituitary–thyroid axis, while TRα null mice have normal thyroid hormone and TSH levels. TRα null mice represent lower heart rate and body temperature (Wikstrom et al., 1998). TRβ null mice defect the auditory function (Forrest et al., 1996).

The ligands capable of binding to TRs are not only endogenous T3 and T4. Several classes of environmental chemicals have a high degree of structural similarity to the THs, and interfere with the binding of THs to TRs (Zoeller, 2005). Polychlorinated biphenyls (PCBs) can bind to TRα and TRβ as antagonists, and disturb TR-mediated transcription (Shiraishi et al., 2003). Recently, it was reported that the polybrominated and polychlorinated diphenylethers, 3,3′,5-trichlorobisphenol A, 3,3′,5-tribromobisphenol A, and 3,3′-dibromobisphenol A, commercially available as fire retardants, have antagonistic activity to TRs (Kitamura et al., 2005; Ghisari et al., 2005; Kudo et al., 2005). TH homeostasis is highly regulated by several protein factors including pituitary peptide hormone TSH and hypothalamus peptide hormone TRH. Because TRs play a key role in TH homeostasis by negative and positive regulation of these protein factors through gene transcription (Chatterjee et al., 1989; Darling et al., 1989; Hollenberg et al., 1995), disruption of TH homeostasis by these exogenous TR ligands is of great concern. Moreover, the differences in function of TRα and TRβ indicate that TH-like activities of chemicals should be assessed by both receptor subtypes individually.

Several *in vitro* bioassays have been developed for the screening of TR ligands. These bioassays are based on quantification of a reporter enzyme as a consequence of nuclear receptor activation in mammalian cells or yeasts (Zoeller, 2005; Jugan et al., 2007; Walfish et al., 1997; Li et al., 2008; Moriyama et al., 2002). These assays have the advantages of being fast and relatively simple. Furthermore, yeast-based bioassays are more cost-effective and easier to handle than mammalian cell-based bioassays. However, all of the reported yeast assays are based on the yeast two-hybrid assay system using the only ligand-binding domain of TRs. We attempted to establish yeast reporter assay systems with "intact" human TRs and coactivator resembling to human cell. In this study, we developed novel reporter yeasts for screening TRα and TRβ ligands with intact TRs and SRC-1, and examined their responses to endogenous THs and chemicals which are suspected to be TR ligands.

2. Materials and methods

2.1. Chemicals

Dimethyl sulfoxide (DMSO), dithiothreitol (DTT), and *o*-nitrophenol-β-D-galactopyranoside (ONPG) were purchased from WAKO Pure Chemical (Osaka, Japan). 3,5-Diiodothyronine (T2), 3,5,3′-triiodothyronine (T3), and 3,5,3′,5′-tetraiodothyronine (T4) were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Tetrabromobisphenol A (TBBPA), tetramethylbisphenol A (TMBPA), and 2-isopropylphenol (IPP) were purchased from Tokyo-Kasei (Tokyo, Japan). *o*-*t*-Butylphenol (OBP) was purchased from Nacalai Tesque (Kyoto, Japan). Restriction enzymes, DNA modification enzymes, and other chemicals were obtained from WAKO Pure Chemical (Osaka, Japan).

2.2. Plasmids

The nuclear receptor expression vector, the coactivator expression vector, and the reporter vector were constructed for the development of TRα and TRβ reporter assays. The nuclear receptor expression vector pUdp6 was constructed as follows. The DNA fragment encoding the yeast *ura3* gene was digested using pESC-ura vector (Invitrogen, Carlsbad, CA) and cloned into pUC18 (Takara Bio, Shiga, Japan). Then, the DNA fragment containing the gal1/gal10 dual directional promoter of YEplac181 (Gietz et al., 1988) was cut and inserted into the vector described above. Two DNA fragments containing the yeast *ADH* terminator and the yeast *cyc1* terminator were amplified by PCR from yeast genomic DNA and inserted downstream of the gal1 and gal10 promoter, respectively. A detailed map of the vector is shown in Fig. 1.

Human TRα, TRβ, and RXRα cDNA were amplified from human mammary gland cDNA (Clontech, Palo Alto, CA) with the primer pairs shown in Table 1. Each cDNA was re-amplified with primers containing a restriction site and/or the yeast ribosomal binding consensus sequence near the initiation codon, and cloned into the multi-cloning site (MCS1 or MCS2) of the pUdp6 vector. The plasmid vector pUdp6-TRα/RXRα contains TRα and RXRα cDNAs in MCS1 and MCS2, respectively. The plasmid vectors pUdp6-TRα and pUdp6-TRβ contain only TRα or TRβ cDNA in MCS1, respectively. To construct the human SRC-1e expression vector, SRC-1e cDNA was amplified by PCR from the cDNA described above and cloned into MCS1 in the pESC-leu vector (Invitrogen, Carlsbad, CA). The reporter vector pRW95-3 includes the TRP-1 selection marker, the yeast Cen6 origin for episomal replication, and the LacZ gene located immediately downstream the cyc1 minimum promoter. The double-stranded oligonucleotide containing the TRE (Table 1) was phosphorylated and inserted upstream of the cyc1 minimal promoter in the pRW95-3 vector (Wolf et al., 1996). Each plasmid vector pYT-DR4×3, pYT-ER6×3, and pYT-IR0×3 contained three copies of DR-4, ER-6, and IR-3 oligonucleotides, respectively. To construct the control reporter vector pYT-cyc expressing β-galactosidase constitutively, the DNA fragment corresponding to the 5′-flanking region of the cyc1 gene was amplified with primer pairs (Table 1), and inserted upstream of the cyc1 minimal promoter of the pWE95-3 vector.

2.3. Yeast strain and transformation

Saccharomyces cerevisiae strain W303a (MAT**a**, *ade*2-1, *trp*1-1, *leu*2-3, *his*3-11, 15, *ura*3-1) was used throughout the experiment. Cells were cultured in synthetic complete medium consisting of 0.67% yeast nitrogen base without amino acids (Difco, Detroit, MI), 2% glucose, and appropriate supplements. Drop-out medium was prepared by excluding the indicated nutrient from the synthetic complete medium. All transformations were performed following the lithium acetate method (Ito et al., 1983). First, the reporter vector was introduced into the yeasts and selected on trp- plates. A single colony was isolated and cultured in trp$^-$ medium. Second, the nuclear receptor expression plasmid was linearized by *EcoRV* and introduced into the yeasts by homologous recombi-

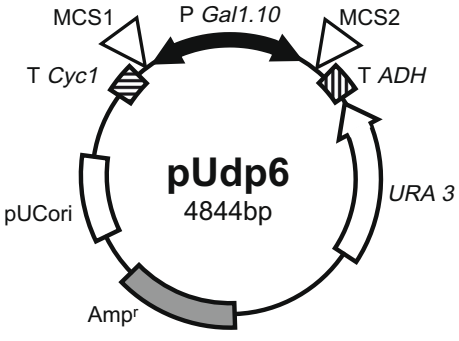

Fig. 1. Plasmid map of pUdp6 vector. Yeast *ura*3 gene (*URA*3), gal1,10 dual directional promoter of YEplac181 (P *Gal*1,10), terminator sequence of yeast alcohol dehydrogenase (T *ADH*) and terminator sequence of yeast cytochrome C (T *Cyc*) were inserted into the parental vector pUC19. TRα or TRβ cDNA were inserted in the multiple cloning site 1 (MCS1) and RXRα cDNA was cloned into the multiple cloning site 2 (MCS2).