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Zebrafish (*Danio rerio*) Thyroid Hormone Receptor α1 Counteracts Retinoic Acid-induced Transcription

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The present study aims to characterize a cDNA encoding zebrafish thyroid hormone receptor α1 (zTRα1) in order to investigate its possible role in the early stage of embryonic development. A mobility shift assay showed that zTRα1 overexpressed in COS7 cells specifically bound to thyroid hormone response element (TRE). In addition, the specific interaction of anti-rat TRα1 antibodies with zTRα1/TRE complexes demonstrated that the cDNA clone encoded zebrafish thyroid hormone receptor α1. Transient cotransfection assays showed that zTRα1 repressed the transcription which was induced by retinoic acid (RA), a well-characterized embryonic morphogen. These results suggest that zTRα1 may be involved in regulating the RA-induced gene transcription during early embryonic development.

Thyroid hormones exert critical effects on normal growth and development as well as a variety of metabolic pathways (Lazar, 1993). They bind to thyroid hormone receptors (TRs) and control the expression of specific target genes in a ligand-dependent manner (Larzar, 1993). TRs, in turn, bind to thyroid hormone response elements (TREs), which are composed of hexamer half-sites [AGGT(C/A)A] with some degeneracy in sequence, number, orientation, and spacing (Barettino et al., 1993). TRs bind to TREs as monomer and homodimer as well as heterodimer forms (Larzar, 1993). Since TRs share a P-box sequence which determines DNA binding specificity with other nuclear hormone receptors such as T3 receptor auxiliary protein (Darling et al., 1991), retinoic acid receptor (RAR) (Evans, 1988), retinoid X receptor (RXR) (Forrest et al., 1991; Fondell et al., 1993; Rhee et al., 1995), and orphan receptors (Glass et al., 1989; Graupner et al., 1989; Hudson et al., 1990), they can heterodimerize with other receptors on composite hormone response elements. The heterodimerization of TRs with other nuclear hormone receptors, for instance, RARs, has been shown to be antagonistic in transient transfection studies (Glass et al., 1989; Graupner et al., 1989; Hudson et al., 1990), suggesting that the two different nuclear receptors can coregulate trasnscription in response to either ligand.

TRα and TRβ RNAs have been detected in *Xenopus laevis* embryos during metamorphosis (Yaoita and Brown, 1990; Baker and Tata, 1990). In particular, TRα is known to be expressed and polysome-associated early in *Xenopus* embryogenesis during blastula, gastrula, and neurula stages of development (Banker et al., 1991) while the thyroid gland is immature, so that the level of thyroid hormone is negligible (for review, see Galton, 1983). Therefore, this early-expressed TRα, if functional, would act independently of T3 or in response to an as yet uncharacterized ligand. On the other hand, RARα and γ subtypes are expressed at relatively high levels during *Xenopus* embryogenesis (Blumberg et al., 1992). Retinoic acid (RA) has been found in *Xenopus* embryos (Durston et al., 1989) and is assumed to be active in controlling normal axis formation during embryogenesis. When *Xenopus* embryos are exposed to micromolar concentrations of RA during blastulation, they are unable to develop normal anterior structures, including cement gland, eyes, forebrain, and anterior hindbrain (Durston et al., 1989; Sive et al., 1990; Papalopulu et al., 1991, Banker and Eisenman, 1993). Since TRα RNA is expressed during the RA-sensitive period of *Xenopus* embryogenesis, it is likely that TRα modulates the RA-induced gene transcriptions in the early embryogenesis. As an initial step to test this hypothesis using zebrafish embryos as an animal model, we studied DNA binding activity and transcriptional activity of the zebrafish TRα1.

Materials and Methods

RT-PCR cloning and isolation of a cDNA encoding zebrafish TRα1

The DNA-binding domain and the T3-binding domain of zTRα1 were amplified using two degenerate oligonucleotides, ztrα3′ and ztrα5′, corresponding to the

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N-terminal region flanking DNA-binding domain and T3-binding domain of TRα1, and containing restriction sites for subsequent cloning (ztrα3′: ACTGCGGATC-CGCAGGGTACATCCCCAGCTA; ztrα5′: ACTGCAAG-CTTGAAGAACTTCGCCGTCTCGAC). Total RNA (2 µg) from 1-32 cell stage embryos was subjected to reverse transcription for 45 min at 42℃, using an oligo (dT) primer, after an annealing step of 5 min at 72℃. An aliquot (10 µl) was then subjected to 35 PCR cycles consisting of 1.5 min at 95℃, 1 min at 55℃ and 1 min at 72℃ in 100 µl of reaction buffer (Promega) supplemented with 1.5 mM $MgCl_2$ and 100 pg of each primer. PCR products of the expected size (600 bp) were extracted by phenol-chloroform, digested with *Bam*HI and *Hind*III, gel-purified and subcloned into pBluescript SKII(-) (Stratagene), and named pzTRα600. The insert of pzTRα600 was random-primed and utilized as a probe to screen the zebrafish heart cDNA library (Stratagene). Inserts of the 6 positive clones from tertiary screening process were isolated and subcloned into the pBluescript SKII(-) vector, and were subjected to DNA sequencing. DNA sequence analyses and comparisons performed using NCBI Genebank data base revealed that a 1.85 Kb-long cDNA was identical to the cDNA encoding zebrafish thyroid hormone recpetor α1 (NCBI accession number: U54796).

Reporter plasmids and expression vectors

Plasmids were propagated in *Escherichia coli* HB 101 and were isolated by alkaline lysis and purfied on Qiagen 500 columns (Qiagen) as described by the manufacturer. The luciferase (LUC) reporter construct pDR4-thymidine kinase (TK)-LUC [pDR4-TK- LUC] was constructed by ligation of an oligonucleotide containing the TRE-DR4 (oligo DR4; 5′-AGCT ACTTA-TTGAGGTCACATGAGGTCAAGTTACG-3′) with 5′-AGTC overhanging ends into a *Hind*III site at the multiple cloning site in front of the TK promoter of a LUC gene-containing plasmid, pT109 (DeWet et al., 1987). The pDR5-TK-LUC reporter was constructed from pDR4-TK-LUC by replacing the spacer consisting of four nucleotides (CATG) between two half sites (AGGTCA), with five nucleotides (CATGC). pcDNA3-zTRα1 and pcDNA3-RARα were produced by inserting the zTRα1 cDNA and RARα cDNA derived from pMT2-RXRα (kindly provided by Dr. S. K. Karathanasis, Lederie Laboratories, Pearl River, NY; Rottman et al., 1991), respectively into the *Eco*RI site of the multiple cloning site of an eukaryotic expression vector, pcDNA3 (Invitrogen).

Electrophoretic mobility shift assay (EMSA)

Whole-cell extracts for EMSA were prepared from COS7 cells mainitained in Dulbecco's modified Eagle's medium plus 10% heat-inactivated (55℃, 1 h) fetal calf serum. The cells were seeded at 10^6 per 100-mm dish 24 h before transfection and were transfected with 10 µg pcDNA3-zTRα1 by the diethylaminoethyl-dextran procedure, essentially as previously described (Widom et al., 1992). After 48 h, cells were scraped from the dishes, pelleted by centrifugation, and resuspended in a buffer containing 20 mM HEPES (pH 7.8), 100 mM KCl, 1 mM dithiothreitol (50 µl/dish). Cell extracts were produced by three freeze-thaw cycles and stored at -70℃. EMSA was performed as described previously (Widom et al., 1992) using the oligo TRE-DR4 (ACTG-AGGTCAACTGAGGTCAACTG) as a probe which was radiolabeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. For supershift assays, appropriate amounts of anti-TRα antibodies, which were previously described (Yen et al., 1992a), were added to reactions and incubated at room temperature for 30 min and then at 4℃ for 2 h.

Cell culture and transient transfections assays

CV-1 cells were grown in Dulbecco's modified Eagle's medium and 10% heat-inactivated (55℃, 1 h) fetal calf serum at 37℃. The serum was stripped of T3 by constant mixing with 5% (wt/vol) AG1-X8 resin (Bio-Rad, Richmond, CA) twice for 12 h at 4℃ before ultrafiltration. The cells were seeded at 10^5 cells per well of a six-well plate (Libron, Flow Laboratories, Inc., McLean, VA) 24 h before transfection. Each well was transfected with expression (60 ng) and reporter (500 ng) plasmids as well as a Rous sarcoma virus-β-galactosidase (RSV-β-Gal) control plasmid (250 ng) (Edlund et al., 1985) by calcium phosphate coprecipitation as previously described (Widom et al., 1992). Cells were grown for 24 h in the presence and absence of T3 (10^{-7} M) before harvesting. Cell extracts were analyzed for both LUC (DeWet et al., 1987) and β-Gal activity. Transcriptional activity is represented by LUC activity, normalized to β-Gal activity.

Results and Discussion

zTRα1 binding to thyroid hormone response element (TRE)

TRs bind various TREs as both homodimers and heterodimers and regulate target gene expression by thyroid, retinoid, and steroid hormones (Larzar, 1993). In an effort to examine the DNA binding specificity of zTRα1 to TRE, zTRα1 was overproduced in COS7 cells through transient transfection and was subjected to mobility shift assays. First of all, we examined the possibility that a COS7 cellular extract contained a detectable amount of endogenous TRα1 interacting with TRE. As shown in Fig. 1, lane 1, 1 µl of untransfected COS7 cellular extract did not produce any bands. In contrast, 1 µl of extract from the pcDNA3-zTRα1-transfected cells bound to TRE-DR4 generated a band (Fig. 1, lane 2). Although TRs bind to TRE as homodimer and monomers, the relative ratio of homodimers to monomers is proportional to the amount of

Fig. 1. Mobility shift and supershift assay of the zTRα1 monomer and homodimer. Reactions containing 0.5 ng [^{32}P]oligo DR4, 1 μl of untransfected COS7 cellular extract (lane 1), or COS7-produced zTRα1 in the presence (lane 3) and absence (lane 2) of 3 μl anti-TRα antibody were analyzed with EMSA using a 4% polyacrylamide gel.

TRs in the EMSA reactions (Yen et al., 1992b). Therefore, it seems that only zTRα1 monodimers were produced due to the low concentration of zTRα1 in the reaction. zTRα1 homodimers were produced in the EMSA which was carried out with a different batch of zTRα1 (Fig. 2, lane 2).

To ensure that the band was composed of zTRα1, we performed the mobility supershift assay with anti-TRα antibody which was originally raised against rat TRα amino acid residues 10-31, the TRα isoform-specific region (Yen et al., 1992b). As shown in Fig. 1, lane 3, the anti-TRα antibodies supershifted zTRα monomers with formation of three additional bands above the band corresponding to zTRα1 monomers. Top two bands were the supershifted homodimers (upper major band) and monomers (lower minor band). The added antibodies increased protein concentration in the supershift reaction and caused a compartmentalization effect to produce more of zTRα1 homodimers (Fig. 2, lane 3). We also confirmed that preimmune serum did not supershift zTRα1 monomers, or homodimers (data not shown). Therefore, it is conclusive that zTRα1 specifically binds to TRE-DR4 as a monomer and a homodimer.

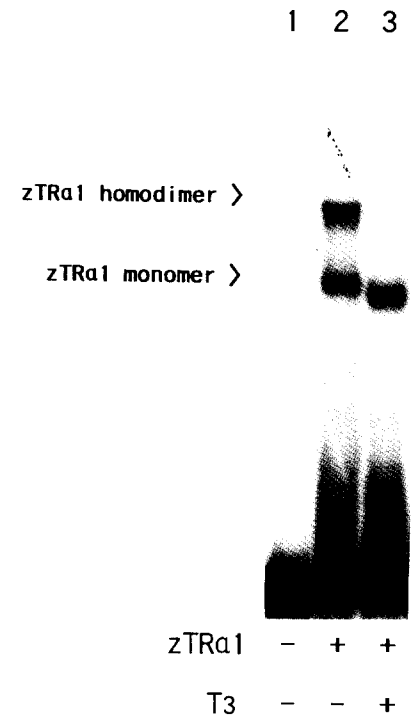

Fig. 2. T3 effect on the zTRα1 on oligo DR4. Autoradiogram from a 4% polyacrylamide gel in the EMSA with 0.5 ng [^{32}P]oligo DR4 and 1 μl untrasfected COS7 cellular extract (lane1) or COS7-produced zTRα1 in the presence (lane 3) and absence (lane 2) of 10^{-7} M T3.

Effect of T3 on dissociation of zTRα1 homodimer from zTRα1/TRE-DR4 complex

TRα, TRβ1, TRβ2 all have been reported to bind T3 with a K_ds range between 10^{-10} and 10^{-11} M (Schueler et al., 1990). T3/TR complexes influence the expression of many genes to varying degrees via TREs, and there could be multiple copies of TREs with which T3/TR complexes interact (reviewed in Larzar, 1993). However, it has also been demonstrated that T3 dissociated TR homodimers from TR/TRE complexes in the reactions for mobility shift assays (Yen et al., 1992b). To examine whether the presence of T3 alters the DNA binding activity of zTRα1 monomer and homodimer, EMSA reaction was carried out in the presence of T3 (10^{-7} M) at room temperature for 15 min. In the absence of T3, there are monomers (lower band) and homodimers (upper band) (Fig. 2, lane 2). However, T3 treatment abolished the homodimer band (Fig. 2, lane 3). In addition, T3 altered the location of the monomer band as it has been known that T3 binds to TR monomers and homodimers and alters their biochemical properties, such as DNA- binding activity, surface charge and conformation (Yen et al., 1992b). Taken together with the EMSA data (Fig. 1), these results demonstrate that zTRα1 is able to bind to TRE-DR4 in a T3-dependent manner.

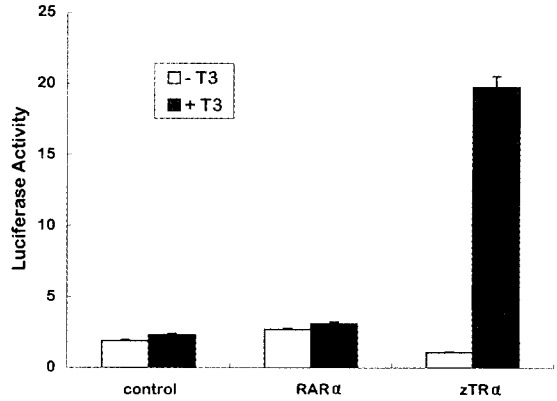

Fig. 3. T3 responsiveness of zTRα1 via the TRE-DR4. The plasmid pDR4-TK-LUC was transiently transfected into CV-1 cells with 60 ng of vectors expressing zTRα1, and RARα, as indicated, in the presence (filled bars) or absence (open bars) of 10^{-7} M T3. Normalized LUC activities are depicted. Results represent the mean of duplicate determinations differing by less than 10% and are representative of five experiments.

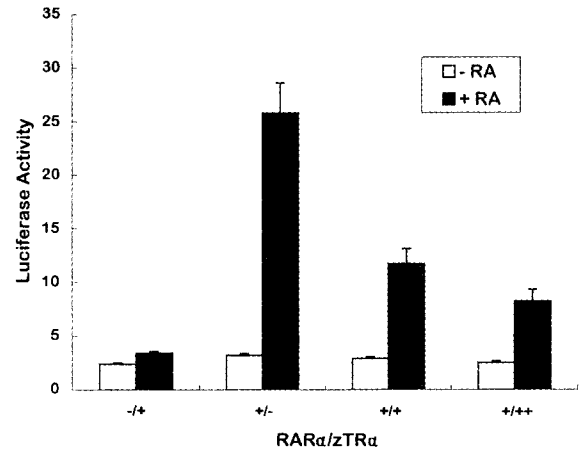

Fig. 4. Effect of zTRα1 on RARα-mediated transcription in the presence (filled bars) and absence (open bars) of RA. The plasmid pDR5-TK-LUC was transiently transfected into CV-1 cells in the indicated amounts (+, 60 ng; ++, 120 ng) of the plasmids expressing zTRα1 and RARα in the presence or absence of 10^{-6} M RA. Normalized LUC activities are depicted. Results represent the mean of duplicate determinations differing by less than 11% and are representative of three experiments.

Down regulation of the RA-induced transcription by zTRα1

The most physiologically relevant property of the cloned TRs is the ability to confer transcriptional responsiveness on TREs when expressed in cells which otherwise do not respond to T3. This requires the ability to bind both T3 and TREs, and has been demonstrated for zTRα1 in this report. We examined the transcriptional responsiveness of zTRα1 to T3 via a specific TRE, the TRE-DR4, which is composed of two DRs (AGGTCA) separated by four nucleotides (CATG). A single copy of TRE-DR4 was inserted proximal to the basal promoter of the TK gene in a vector pT109 containing the LUC gene (pDR4-TK-LUC). pDR4-TK-LUC was cotransfected into CV-1 cells with vectors expressing zTRα1 (pcDNA3-zTRα1), RARα (pcDNA3-RARα), and β-Gal (pRSV-β-GAl) in the presence and absence of T3 as shown in Fig. 3. LUC activity in extracts from the cells was determined and normalized with respect to the corresponding β-Gal activity. As shown in Fig. 3, unliganded-zTRα1 repressed the basal transcription by 57% (Graupner et al., 1989), whereas liganded-zTRα1 increased transcription as much as 18-fold in comparison with unliganded-zTRα1. As expected, RARα alone neither down-regulated basal transcription nor activated transcription in the presence of T3 (Fig. 3). These results demonstrated that zTRα1 was able to transactivate transcription via the TRE-DR4 in response to T3.

TRs heterodimerize with other nuclear hormone receptors to extend their involvement in regulating gene transcription (Glass et al., 1989; Graupner et al., 1989; Hudson et al., 1990). For instance, in the absence of retinoic acid (RA), the RAR also inhibits T3-responsive gene expression, presumably due to interference with TRE binding and/or heterodimer formation (Umesono et al., 1988; Graupner et al., 1989). In the presence of RA, the RAR activates transcription from TRE-containing genes, consistent with its ability to bind to the TRE (Umesono et al., 1988; Bedo et al., 1989). To examine whether zTRα1 is involved in regulating retinoic acid-induced gene transcription, we performed transient cotransfection assays using pcDNA3-zTRα1, pcDNA3-RARα, pRSV-β-Gal and pDR5-TK-LUC: the TRE-DR4 in pDR4-TK-LUC was replaced with a DR5 which has been known as a RA-response-element (RARE) (Umesonono, 1988) as shown in Fig. 4. As expected, zTRα1 alone did not alter transcription while liganded-RARα stimulated via RARE as much as 8-fold in comparison with unliganded-RARα. Cotransfection of pcDNA3-zTRα1 with pcDNA3-RARα reduced the RA-induced transcription by 55% (Fig. 4). Cotransfection of two-fold excess of pcDNA3-zTRα1 with pcDNA3-RARα further reduced the LUC activity by 68%, indicating that zTRα1 down-regulated the RA-mediated transcription. If this downregulation happens in early embryos, it would be of interest to ask whether inhibition of endogenous zTRα1 expression during early embryogenesis causes a teratogenic effect as does the RA-treatment in early embryos.

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