Functional Significance of a Truncated Thyroid Receptor Subtype Lacking a Hormone-Binding Domain in Goldfish

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Thyroid hormones are important mediators of growth and development in vertebrates and act by binding to a specific family of thyroid receptors (TRs). The TRs belong to the nuclear receptor superfamily, with two conserved regions, a DNA binding domain and a ligand binding domain (LBD). We recently demonstrated the presence of four TR subtypes in goldfish, two with complete DNA binding domains and LBDs (TRα-1 and TRβ) and two novel forms including a transcript resembling TRα with variation in the LBD as well as a TRα-truncated (TRα-t) form lacking an LBD. To study the functional significance of TR subtypes, we first investigated the regulation of hepatic goldfish deiodinase type 3 (D3) by T3 and validated a bioassay in which D3 gene expression is up-regulated significantly in vivo and in vitro. Using short interfering RNA, TRα-1, TRβ, or TRα-t was specifically knocked down and thyroid hormone-induced D3 gene expression was measured. Short interfering RNA against TRα-1 or TRβ reduced the T3 induction of deiodinase gene expression to 50% or less than 25% of control (T3 treated) cells, respectively. Knocking down TRα-t alone, however, increased D3 expression 500-fold supporting the hypothesis that TRα-t plays a modulatory role in thyroid hormone-induced gene expression. Our results provide important insight into thyroid receptor biology in goldfish and a framework for the better understanding of thyroid receptor function in all vertebrates. (Endocrinology 149: 4702–4709, 2008)

First Published Online May 29, 2008

Abbreviations: D3, Deiodinase type 3; DMSO, dimethylsulfoxide; HDAC, histone deacetylase; LBD, ligand binding domain; QPCR, quantitative (real time) PCR; RACE, rapid amplification of 3’ cDNA ends; siRNA, short interfering RNA; TR, thyroid receptor; TRE, thyroid response element; TRα-t, TRα-truncated; UTR, untranslated region. Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.
Materials and Methods

Animals

Male and female goldfish (Carassius auratus), ranging from 8 to 12 cm in length, were killed in accordance with the principles and guidelines of the Canadian Council of Animal Care.

Primary hepatocyte culture

Goldfish livers of mixed sex were manually diced washed in buffer followed by treatment with collagenase, trypsin, and deoxyribonuclease at 28 C with gentle shaking for 30–45 min and then filtered through a 40-μm nylon sieve. Cells were washed and loaded onto a Percoll (GE Life Sciences, Piscataway, NJ) gradient (20–40–60%) and spun at 1200 rpm for 30–45 min. Hepatocytes were isolated, washed, and plated on 24-well plates at a density of 750,000 and incubated in 1 ml of phenol red free M199 with penicillin and streptomycin.

Cloning of goldfish D3

All primers used are listed in supplemental Table 1, published as supplemental data on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org. RNA extraction, RT-PCR, and rapid amplification of 3′ cDNA ends (RACE) was carried out as reported elsewhere (22). 5′ RACE was performed using the SMART kit by CLONTECH (Palo Alto, CA). Sequences were aligned and analyzed using National Center for Biotechnology Information (Bethesda, MD) and Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin/) web pages.

In vivo regulation of D3 by T3

Goldfish were injected ip with 0, 2.5, 25, and 250 ng of T3 per fish. Controls were injected with vehicle dissolved saline. Semiquantitative RT-PCR was performed as described previously (22, 23). As an internal control, 18S rRNA was also amplified for each sample.

In vitro regulation of D3

Goldfish hepatocytes were treated with T3 at doses of 0, 0.5, 5, and 50 ng/ml for 5 h. Doses of 0.5 and 5 ng/ml resemble physiologic plasma levels of T3 reported in other studies (24, 25). Control cells were treated with vehicle. Quantitative (real time) PCR (QPCR) was performed as described below. Separate experiments were carried out to determine the nature of the D3 induction by T3 and the involvement of HDAC. Cells were treated with either T3 (50 ng/ml), an inhibitor of protein translation (cycloheximide, 15 μg/ml), an inhibitor of transcription (actinomycin-D, 10 μg/ml), an HDAC inhibitor [4-dimethylamino-N-(6-hydroxy carbamoyl)hexyl], benzamide, 1 μM (Calbiochem, La Jolla, CA); analog of trichostatin A that potently inhibits histone deacetylases (26–28)], or both T3 and an inhibitor. A dose of 1 μM was chosen for the HDAC inhibitor because this is a 10-fold higher concentration than the IC50 for maize and would likely result in a near compete HDAC inhibition in goldfish. Differentiation was induced and proliferation was inhibited in murine erythroleukemia cells at about 2 μM. For the HDAC experiments, two controls were used, one treated with the vehicle for the HDAC inhibitor [dimethylsulfoxide (DMSO)] and the other treated with the vehicle for T3 (NaOH).

RNA interference

siRNA specifically targeting each of the goldfish TR subtypes in addition to one siRNA, which would target all of the TR subtypes, were synthesized and purified (University of Calgary Core DNA Services) (supplemental Table 2). Cells were transfected using Exgen (Fermentas, Hanover, MD). Optimization experiments revealed that 180 pmol of siRNA was the minimum concentration for effective knockdown, and the knockdown lasted for at least 48 h (as determined by QPCR). To control for nonspecific siRNA effects such as the interferon response, Block-It, a fluorescein-labeled double stranded RNA oligo (Invitrogen, Carlsbad, CA), which does not share homology to any known sequence was used (referred to as scrambled). Thirty-six hours after transfection, cells were treated with either T3 (50 ng/ml) or the HDAC inhibitor for 5 h. To ensure specificity of siRNA, the expression of all TRs was measured for each experiment (supplemental data).

QPCR

Primers were listed in supplemental Table 1. All resulted in one amplon (as determined by melt curve and gel electrophoresis analysis) and had an efficiency greater than 90%. As an internal control, β-actin was also amplified as described in (23). A iCycler IQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA) was used with the following conditions per well: 0.5 μl cDNA, 0.26 μM of each primer, 0.2 mM deoxynucleotide triphosphates, Sybr green, and Taq polymerase in buffer [10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.4 mM MgCl2, 20 nM fluorescein] to a total volume of 25 μl. Cycling was as follows: 3 min at 94 C followed by 30–50 cycles of 10 sec at 94 C and 40 sec at 55 C (TRs and actin) or 57.6 C (D3). Each experimental group was run in duplicate or triplicate to ensure consistency. Results were calculated using the relative 2^-ΔΔCt method, with β-actin as an internal control. Control (un-treated) values were set at 1.

Determination of absolute TR mRNA levels

QPCR was used to determine the absolute mRNA levels for each TR subtype by comparison with standard curves generated in a similar way to RoseMeyer et al. (29). Briefly, specific PCR product, generated as described for QPCR, was gel-purified and quantified (OD260), and used to create a dilution series (standard curve). Ten separate liver cDNA samples were run alongside and their expression calculated based on the standard curve.

Statistics

The results (either raw or Ln transformed) were analyzed by one-way ANOVA followed by the Student Newman Keuls multiple comparison test (significance if P < 0.05).

Results

Identification of goldfish D3 sequences

The complete cDNA sequence for goldfish D3 was determined. The nucleic acid sequence contained a TGA codon,
which would code for a selenocysteine (U). RACE revealed two forms of goldfish D3 (named type a and type b). Both are identical with alternate untranslated regions (UTRs) (supplemental Fig. 1). Type a has 1116 bp and type b has 1182 bp. A putative selenocysteine insertion sequence has been identified. Both end code for the same 274 amino acid protein, with a selenocysteine that shares high homology with other species (supplemental Table 3). The sequence has been deposited in the National Center for Biotechnology Information GenBank (accession no. D3a: EF190704 and D3b: EF190705).

Effect of T3 on D3 expression, in vivo

Male and female fish showed very similar D3 expression in either control or T3-treated fish (data not shown). Therefore, sexes were combined for data analysis. D3 expression was very low in untreated fish but increased dramatically in a dose-dependent manner after treatment with T3 (Fig. 1). After only 12 h after treatment, D3 expression was 740 times and more than 1000 times higher in fish treated with 25 and 250 ng of T3, compared with control. D3 expression remained significantly higher in fish treated with 250 ng of T3 after 24 h. Whereas a pattern of increased D3 expression with dose of T3 was apparent after 36 h, these changes were no longer significantly different from control levels.

D3 expression is up-regulated by T3 in vitro and is repressed by histone deacetylase

T3 up-regulated D3 expression in vitro in a dose-dependent manner (Fig. 2A). A dose of 5 ng/ml lead to a significant increase in D3 expression (>50-fold), whereas a dose of 50 ng/ml lead to an increase greater than 175-fold. The translation inhibitor, cycloheximide, had no effect, whereas the transcription inhibitor (actinomycin-D) inhibited the induction of D3 by T3 (Fig. 2B). In a separate experiment, treatment with T3 (50 ng/ml) increased D3 expression by greater than 550-fold. Inhibiting HDAC increased basal levels of D3 by 189-fold, or 34% of T3-induced levels (Fig. 2C). A combination of the HDAC inhibitor and T3 had a synergistic effect, increasing D3 expression by 1182-fold (expected additive expression would be 741-fold higher than control).

TRα-t expression is more abundant than the other subtypes

When investigating the basal absolute mRNA levels, it was found that TRα-t had more than 3-fold the number of copies, compared with TRα-1 and TRα-2 (7.4, 2.0, and 2.2 × 10^5 copies/μg of total RNA, respectively) (see Fig. 6, inset). TRα-t expression was found to be twice as high as TRβ (3.7 × 10^5 copies/μg RNA).

Knockdown of TRα-1 reduces T3 induction of D3 by half

siRNA targeting TRα-1 decreased its expression to less than 5% of its control level, as determined by QPCR (Fig. 3). Both TRα-1 and D3 expression remained unchanged between control groups, groups treated with siRNA with for β-actin and with respect to control levels (mean ± SEM). Different letters denote a significant difference (P < 0.05, n = 4–6/group).
no homology (scrambled), and siRNA against TRα-1. T3 up-regulated D3 expression by greater than 300-fold in either control cells or cells treated with scrambled siRNA. Only half of this response was observed in cells treated with siRNA against TRα-1. siRNA against TRα-1 did not effect the expression of the other TR subtypes (supplemental Fig. 2A). siRNA against TRα-2 was unsuccessful (data not shown).

Knockdown of TRβ reduces T3 induction of D3 by 85%

siRNA against TRβ significantly reduced TRβ expression by more than 95% (Fig. 4). Scrambled siRNA had no signif-

icant effect on TRβ or D3 expression. Treatment with T3 significantly up-regulated D3 by more than 200-fold in both control cells and cells treated with scrambled siRNA. Whereas treatment with siRNA against TRβ had no significant effect on the basal expression of D3, cells treated with it exhibited less than 15% of the T3-induced response when compared with control cells treated with scrambled siRNA. siRNA against TRα-2 did not effect the expression of the other TR subtypes (supplemental Fig. 2B).

siRNA against TRα-t results in increased D3 expression and synergistically increases T3 induction of D3

siRNA against TRα-t significantly reduced TRα-t expression (Fig. 5). Scrambled siRNA had no effect on TRα-t or D3 expression. Treatment with only T3 induced D3 expression by about 500-fold. Treatment with siRNA against TRα-t alone increased D3 expression by greater than 100-fold. This induction was still significantly lower
than induction of control cells by T3. Treatment with siRNA against TRα/t followed by T3 up-regulated D3 by greater than 900-fold, which is almost twice that of T3 treated control cells and greater than the additive effect of siRNA against TRα/t and T3 treatment alone. siRNA against TRα/t did not affect the expression of the other TR subtypes (supplemental Fig. 2C).

**HDAC inhibition does not increase D3 expression to the same extent in cells treated with siRNA against TRβ**

Treatment with the HDAC inhibitor alone resulted in an induction of D3 greater than 225-fold, whereas treatment with T3 increased expression by greater than 700-fold (Fig. 6). Treatment with siRNA against TRβ followed by the HDAC inhibitor resulted in only a 8-fold increase, significantly lower than HDAC inhibitor treatment alone.

**Knockdown of TRα-t does not alter HDAC-induced D3 expression**

Treatment with the HDAC inhibitor, T3, siRNA against TRα-t, or combinations of these all lead to significant increases in D3 expression when compared with control levels (Fig. 7). Treatment with the HDAC inhibitor alone resulted in a similar increase in D3 expression as cells treated with siRNA against TRα-t followed by the HDAC inhibitor.

**Knockdown of all known TR subtypes results in increased D3 expression**

An siRNA targeting all known TR subtypes (siAll) resulted in at least a 75% knockdown (Fig. 8A). This knockdown was sufficient to increase D3 levels by 154-fold, which is significantly higher than untreated control cells and cells treated with siRNA against TRα-t (Fig. 8B). Treatment of knocked-down cells with T3 did not have a significant effect, compared with knockdown alone. Treatment with siRNA against TRα-t
followed by T₃ significantly up-regulated D3 over all groups except T₃ alone.

**Discussion**

The present study provides novel information on functional significance of TR subtypes, using goldfish as experimental model. Goldfish is a cyprinid species, which is an important comparative model as well as being significant in commercial aquaculture. Previous studies in goldfish provided information on four TR subtypes expressed in the liver (22). In an effort to determine the functionality of the goldfish TRs, including a novel type lacking a LBD, we used a physiologically relevant bioassay approach. Based on information available on amphibian and other fish species (30–32), we validated D3 response as a robust marker for T₃ action in goldfish. We identified the full cDNA sequence for two types of D3, identical in sequence except with alternative 3'UTRs. D3 sequence aligned well to other species and contained a TGA codon within the open reading frame (encoding a selenocysteine), lending support to its identity as deiodinase. Analysis of the 3'UTRs of D3 revealed a putative selenocysteine insertion sequence, which has been shown in other vertebrates to interact with the ribosome and permit a selenocysteine to be coded for, rather than a stop codon (for review see Ref. 33). An alternative 3'UTR for D3 has also been described in the rainbow trout (Oncorhynchus mykiss) (31). The primers used to determine D3 expression in this study were not able to distinguish between the two types of D3.

Consistent with our results for goldfish, hepatic D3 induction by T₃ has been reported in amphibians, Nile tilapia, and rainbow trout (30–32, 34). It is likely that the up-regulation observed in vivo is to a large extent due to direct action of T₃ because treatment of isolated goldfish hepatocytes in vitro also increased D3 expression, and a translation inhibitor (cycloheximide) had no effect on this induction. In related work, we found that the amplitude of D3 induction by T₃ was seasonally dependent on the reproductive stages of goldfish (our unpublished data), which may in part explain the observed variation in T₃-induced response in different experiments. We demonstrated that as in mammals, in the absence of ligand, the TRs actively repress gene transcription, in part via HDAC activity, because an HDAC inhibitor also increases D3 expression.

The T₃ induction of D3 was diminished in cells treated with siRNA against either TRα-1 or TRβ. It appears that TRβ may play a larger role in the T₃-mediated expression of D3 because when TRβ was knocked down, T₃ induction of D3 was less than 25% of induced control cells, compared with about 50% when TRα-1 was knocked down.

Interestingly, knocking down TRα-t increased basal levels of D3 expression. This indicates that TRα-t normally suppresses D3 transcription. Additionally, treatment of cells lacking TRα-t with T₃ was found to increase D3 expression, further supporting our hypothesis that TRα-t modulates normal thyroid activity. Previous studies on TRs lacking functional LBDs have been limited to mammalian TRα-2. The
dominant-negative activity of TRα-2 has been demonstrated for rat (16, 17, 35) and human (36). However, these studies generally rely on overexpression of TRα-2 and reporter assays. As such, the mechanism of action for TRα-2 is not fully understood. Initial studies indicated that TRα-2 competes with functional TRs for thyroid response elements (TREs) to exert its dominant-negative activity (37, 38). However, TRα-2 retains its antagonistic activity, even when binding to TRE is prevented through mutation in the DNA-binding domain. Here it is proposed that TRα-2 may quench other factors such as coactivating proteins (39). Additionally, it has been shown that the nature of TRE is important for the specificity of TRα-2. If TRα-2 heterodimerizes with the retinoid-X receptor, then it has the highest affinity for direct repeat response elements but very poor affinity for palindromic or inverted palindromic elements (40). Our study offers the first evidence that a TR lacking a LBD does antagonize normal thyroid activity, using primary cells with endogenous levels of all TRs using a physiologically relevant thyroid-responsive gene.

Whereas the dominant-negative activity of mammalian TRα-2 is relatively small (41), truncation of its C terminus increased its affinity for DNA and its dominant-negative effects (37, 42). Because goldfish TRα-t is also a truncated form of TR, it is possible that it too binds DNA with high affinity and may help explain the strong antagonistic effects observed. This proposed competition between TRα-t and the full-length TRs must still be formally tested.

Based on the fact that an HDAC inhibitor increased D3 expression, it would be expected that if either TRα-1 or TRβ were knocked down, there would be a relief in active repression and a corresponding increase in D3 should be observed. However, this was not the case. D3 expression in cells treated with siRNA against either TRα-1 or TRβ remained at basal levels. There are at least three possible explanations for this: 1) the knockdown at the protein level was not sufficient to alleviate repression, 2) the other functional TR replaces the knocked-down TR and compensates for it, or 3) TRα-t or TRα-2 replaces the knocked-out TR and repress transcription. The first scenario is unlikely due to the large differences observed in D3 induction by T3 between control cells and siRNA-treated cells (Figs. 3 and 4). If TRα-1 replaces the knocked-out TRβ as scenario two suggests, then it would be expected that treatment of these cells with an HDAC inhibitor would alleviate repression to the same extent as control cells treated with the HDAC inhibitor. This was not the case; whereas there was a small induction of D3 by an HDAC inhibitor, its expression was significantly diminished, compared with control cells of the same treatment (Fig. 6). Furthermore, when TRα-t was knocked down and treated with an HDAC inhibitor, D3 levels resembled the additive responses of TRα-t knockdown alone and HDAC inhibition alone (Fig. 7). Additionally, we found that TRα-t mRNA levels are higher than either of the other subtypes (Fig. 6, inset), supporting a competition-based model. That leaves scenario 3 as the most likely: either TRα-2 or TRα-t replaces the knocked-down TR and represses D3 transcription in a HDAC-independent mechanism. In further support of an HDAC-independent mechanism, both TRα-2 and TRα-t lack the residues in the LBD of TRs normally associated with interacting with corepressors (43). In final support of this model, when all TRs are knocked down, there is a relief of repression and an increase in D3 transcript in the absence of ligand. This relief is significantly higher than the relief seen when TRα-t alone is knocked down.

Taken together, we propose a model for the action of TRs in the goldfish liver including three possible configurations on the deiodinase promoter: TRα-1 + nuclear receptor dimerization partner, TRβ + nuclear receptor dimerization partner, or TRα-truncated + potential dimerization partner. In the absence of ligand, all three configurations repress transcription. TRα-1 and TRβ combinations repress via histone deacetylase. In the presence of ligand, corepressors are lost and coactivators are recruited to TRα-1 and TRβ configurations, leading to increased transcription, whereas the presence of TRα-truncated continues to repress transcription of TRα-1 and TRβ mediated genes.

In addition to characterizing the goldfish deiodinase-3 gene and its regulation by T3, we used siRNA to knock down endogenous receptor levels, demonstrating clear changes in a cellular physiological response. Our results provide strong support for the hypothesis that a thyroid receptor molecule lacking the ligand binding domain plays a modulatory role in thyroid hormone-induced gene expression, using goldfish as an experimental model. These findings provide important insight into thyroid receptor biology in goldfish and a framework for the better understanding of thyroid receptor function in all vertebrates.

Acknowledgments

We thank Dr. Dave D. Hansen for the use of his equipment and Flora Pang for her help in developing a goldfish hepatocyte cell culture protocol.

Received January 24, 2008. Accepted May 16, 2008.

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This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (to H.R.H.).

Disclosure Summary: The authors have nothing to disclose.

References

2. Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889–895
7. Lazar MA, Berodin TJ, Harding HP 1991 Differential DNA binding by monomeric, homodimeric, and potentially heteromeric forms of the thyroid hormone receptor. Mol Cell Biol 11:5005–5015
8. Mengeling BJ, Pan F, Privalsky ML 2005 Novel mode of deoxyribonucleic acid recognition by thyroid hormone receptors: thyroid hormone receptor β-

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forms can bind as trimers to natural response elements comprised of reiterated half-sites. Mol Endocrinol 19:35–51


11. Harvey CB, Williams GR 2002 Mechanism of thyroid hormone action. Thyroid 12:441–446


15. Lazar MA, Hodin RA, Darlington, DS, Chin WW 1988 Identification of a c-erbA α-related protein which binds deoxyribonucleic acid but does not bind thyroid hormone. Mol Endocrinol 2:903–901


23. MacKenzie DS, Sokołowska M, Peter RE, Breton B 1987 Increased gonadotropin levels in goldfish do not result in alterations in circulating thyroid hormone levels. Gen Comp Endocrinol 67:202–213


28. Becker KB, Schneider MJ, Davey JC, Galton VA 1995 The type III 5-deiodinase in Rana catesbeiana tadpoles is encoded by a thyroid hormone-responsive gene. Endocrinology 136:4424–4431

29. Bres O, Plohmam JC, Eales JG 2006 A cDNA for a putative type III deiodinase in the trout (Oncorhynchus mykiss): influence of holding conditions and thyroid hormone treatment on its hepatic expression. Gen Comp Endocrinol 145:92–100


34. Lazar MA, Hodin RA, Chin WW 1989 Human carboxy-terminal variant of α-type c-erbA inhibits trans-activation by thyroid hormone receptors without binding thyroid hormone. Proc Natl Acad Sci USA 86:7771–7774

35. Katz D, Lazar MA 1993 Dominant negative activity of an endogenous thyroid hormone receptor variant (α2) is due to competition for binding sites on target genes. J Biol Chem 268:20904–20910


37. Liu RT, Suzuki S, Miyamoto T, Takeda T, Ozata M, DeGroot JJ 1995 The dominant negative effect of thyroid hormone receptor splicing variant α2 does not require binding to a thyroid response element. Mol Endocrinol 9:95–96


40. Katz D, Berrodin TJ, Lazar MA 1992 The unique C-termini of the thyroid hormone receptor variant, c-erbA α2, and thyroid hormone receptor α1 mediate different DNA-binding and heterodimerization properties. Mol Endocrinol 6:805–814


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