Negative regulation by thyroid hormone receptor requires an intact coactivator-binding surface

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Thyroid hormone (TH) action is mediated by TH receptors (TRs), which are members of the nuclear hormone receptor superfamily. In vitro studies have demonstrated that TR activity is regulated by interactions with corepressor and coactivator proteins (CoRs and CoAs, respectively). TH stimulation is thought to involve dissociation of CoRs and recruitment of CoAs to the liganded TR. In contrast, negative regulation by TH is thought to occur via recruitment of CoRs to the liganded TR. The physiological role of CoAs bound to TRs, however, has yet to be defined. In this study, we used gene-targeting techniques to mutate the TR-β locus within its activation function–2 (AF-2) domain (E457A). This mutation was chosen because it completely abolished CoA recruitment in vitro, while preserving normal triiodothyronine (T3) binding and CoR interactions. As expected, TH-stimulated gene expression was reduced in homozygous E457A mice. However, these animals also displayed abnormal regulation of the hypothalamic-pituitary-thyroid axis. Serum thyroxine, T3, and thyroid-stimulating hormone (TSH) levels and pituitary Tshb mRNA levels were inappropriately elevated compared with those of WT animals, and L-T3 treatment failed to suppress serum TSH and pituitary Tshb mRNA levels. Therefore, the AF-2 domain of TR-β is required for positive and, paradoxically, for negative regulation by TH in vivo.

Introduction

The synthesis of thyrotropin-releasing hormone (TRH), produced in the hypothalamus, and the α and β subunits of thyrotropin (thyroid-stimulating hormone [TSH]) in the anterior pituitary is inhibited at the transcriptional level by thyroid hormone (TH) (1). Negative regulation of gene expression by triiodothyronine (T3) is critical in the control of the hypothalamic-pituitary-thyroid (H-P-T) axis, and this effect is mediated by the β isof orm of the TH receptor (TR) (2–6).

TH action is mediated by different isoforms of the TR, which are members of the nuclear receptor superfamily of ligand-modulated transcriptional factors (7). Two genes, by alternative splicing and alternative transcriptional site utilization, express all known ligand-binding TR isoforms, TR-α1, TR-β1-3. Mice deficient in either TR-α or TR-β isoforms display unique phenotypes, which suggests that different TR isoforms have unique regulatory roles (4–6, 8). On genes with a positive TH response element (pTRE) and in the absence of hormone, TR associates with corepressor proteins (CoRs), such as silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear CoR (NCoR), which assemble a complex that inhibits gene transcription. In the presence of ligand, this CoR complex dissociates and is replaced by a coactivator protein (CoA) complex that then activates transcription at the promoter (7). In contrast, at a negative TH response element (nTRE), activation of transcription occurs in the absence of ligand, when, paradoxically, TH might be expected to be in a complex with CoRs and not CoAs (7).

Several models have been suggested to explain the mechanism of negative regulation by TH. On the TRH and TSH subunit genes, TR DNA binding has also been reported to be necessary for negative regulation by TH (9–12). Some investigators, however, have suggested models wherein TR DNA binding is not required for negative regulation. In a report on one such model, it was proposed that both the DNA-binding domain of TR-β and direct DNA binding of TR within the target gene were not necessary for TR-mediated transrepression (13). This mechanism has been proposed to explain negative regulation of the Tsha promoter, in which an nTRE in the proximal promoter has not been clearly identified (14, 15). A DNA binding–independent mechanism similar to that for the nuclear hormone receptor action has also been proposed for the glucocorticoid receptor (16). In contrast, our group has demonstrated, using a well-defined TR DNA-binding P box mutant (GS125), that DNA binding by TR is required for negative regulation both in vitro (17) and in vivo (18). Thus, models of negative regulation must accommodate a TR bound to a target gene in the absence and presence of TH.

Given the importance of DNA binding for negative regulation by TH, we next sought to determine what additional functions of the TR are required. The aim of this study was to create a transgenic

Nonstandard abbreviations used: AF-2, activation function–2; CoA, coactivator protein; CoR, corepressor protein; Dio1, 5′-deiodinase type I; Gsta, glutathione-S-transferase; H-P-T, hypothalamic-pituitary-thyroid; LoI/PTU diet, low iodine diet containing 0.15% 5-propyl-2-thiouracil; L-T3, L-triiodothyronine; MMI, methimazole; NCoR, nuclear CoR; nTRE, negative TRE; pTRE, positive TRE; RIP1-40, receptor-interacting protein 140; RXR, retinoid X receptor; SMRT, silencing mediator for retinoid and thyroid hormone receptors; SRC-1, steroid receptor CoA-1; T3, triiodothyronine; T4, thyroxine; TR, thyroid hormone receptor; TSH, thyroid-stimulating hormone; TH, thyroid hormone; TSHb, thyroid-stimulating hormone–binding protein; TSHr, thyroid-stimulating hormone receptor.

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A mouse model in which the CoA-binding site on TR-β was disrupted, while maintaining CoR and T3 binding activity. Results in the E457A mutant mice demonstrated that an intact CoA-binding surface is important for positive regulation in vivo. Surprisingly, this same domain is also essential for negative regulation of gene expression in the H-P-T axis and liver.

Results

To address the importance of the CoA-binding surface on TR-β in vivo, we utilized a helix 12 mutant of TR-β (E457A; Figure 1A) with normal T3 binding affinity (19, 20). When tested in gel mobility shift assays, it displayed normal affinity for both pTREs and CoR (NCoR) but was unable to bind the CoA, steroid receptor CoA-1 (SRC-1), and receptor-interacting protein 140 (RIP140), after T3 treatment (Figure 1, B–D). Moreover, this mutant was almost completely defective in mediating negative regulation by TH and was partially impaired in mediating positive gene regulation (Figure 1E).

The E457A mutation was then introduced into the Thrb locus via homologous recombination in ES cells (exon 7, Figure 2A). Homologous recombination in ES clones was confirmed by Southern blot analysis and PCR (data not shown). Chimeric males were derived from 2 targeted ES cell clones and transmitted the mutation through crosses with C57BL/6 females; heterozygous mutant mice were interbred. The genotype of F1 and subsequent generations was identified by Southern blot analysis (Figure 2B).

To establish that TR-β was mutated in E457A mice, we first examined TR-β transcripts by performing RT-PCR analysis on total RNA extracted from adult mouse pituitary tissue of WT (TR-βWT/WT) and E457A mutant heterozygous (TR-βE457A/WT) and homozygous (TR-βE457A/E457A) animals (Figure 2C) using primers described in Methods. PCR products from TR-β (E457A) knock-in mice were the same size as those from WT animals (Figure 2C). The presence of E457A mutation was confirmed by subsequent sequencing of the cloned PCR product (Figure 2D). E457A mutant TR-β mRNA was expressed at the same level as WT Thrb mRNA, as indicated by real-time PCR analysis of total pituitary RNA (Figure 2E), which indicated that the mutation did not affect expression from the Thrb locus. Moreover, this mutation did not affect Thrα expression in the same animals. E457A knock-in mice were born with no gross anatomic or functional abnormalities and were viable through adulthood. Both males and females displayed normal fertility.

Serum thyroxine (T4) and T3 levels were higher in TR-β (E457A) knock-in mice, as shown in Figure 3. The TR-βE457A/E457A mice displayed T3 and T4 levels up to 5 times higher than those in WT mice (P < 0.001; Figure 3, A and B). Heterozygous animals displayed a 2-fold increase in T3 levels compared with those in
WT mice ($P < 0.001$); and $T_3$ values were higher than those of control animals, although the difference was not statistically significant (Figure 3B). Similar results were obtained in both male and female mice (data not shown). Basal serum TSH levels were also elevated in the homozygous mice, reaching up to 9 times those observed in WT animals ($P < 0.001$; Figure 3C). $Tshb$ subunit mRNA levels were similarly elevated at baseline in the homozygous animals. Although individual heterozygous animals displayed elevated TSH values, the mean level was not significantly different from that of WT animals (Figure 3C).

Serum TSH and $Tshb$ subunit mRNA levels (Figure 3D) were not suppressed in TRβ(E457A/E457A) mice, despite elevated circulating TH concentrations, which indicates that they have central TH resistance — resistance at the level of the pituitary and/or hypothalamus. In addition to central TH resistance, the TRβ(E457A/E457A) and TRβ(E457A/WT) mice also displayed peripheral tissue resistance. Glutathione-S-transferase ($Gsta$) mRNA levels in the liver, which are normally suppressed in the presence of elevated TH levels (21), were elevated in homozygous mutant mice (Figure 3E).

Histological comparison of the thyroid glands in TRβ(E457A/E457A) mice with those in WT mice revealed an overall enlargement, as predicted from the serum hormone levels (Figure 4). Despite normal body weight, the thyroid weight of TRβ(E457A/E457A) mice was approximately 3-fold greater than that of control animals ($P < 0.001$; Figure 4). Thyroid weight of heterozygous mice was also increased, but to a lesser extent and was consistent with the small increase in serum TSH concentration found in these animals.

To investigate the further the molecular mechanism of abnormal regulation of the H-P-T axis in TRβ(E457A/E457A) animals, we examined mice in both a TH-deficient and an excess state. After 35 days of a low iodine diet containing 0.15% 5-propyl-2-thiouracil (LoI/PTU diet) supplemented with methimazole (MMI) in the water, all animals exhibited markedly elevated TSH levels (> 15,000 mU/l; Figure 5A) and undetectable serum $T_3$ levels (data not shown). L-T$_3$ treatment was then administrated by daily subcutaneous injections for 5 days at 3 escalating concentrations. Unlike in control and heterozygous animals, serum TSH and $Tshb$ subunit mRNA levels in TRβ(E457A/E457A) mice were not suppressed completely even at the highest $T_3$ dose, which indicated markedly reduced sensitivity to negative feedback regulation by TH (Figure 5, A and D). At the end of the middle dose period, TRβ(E457A/WT) mice also exhibited elevated TSH levels compared with WT animals, although this difference did not achieve statistical significance. Unlike serum TSH and TSH-$β$ subunit mRNA levels, TSH-$α$ subunit RNA levels were suppressed in all 3 groups after administration of the higher dose of L-T$_3$ (Figure 5C). However, the degree of suppression in homozygous animals was much less than that observed in WT mice.

To investigate the effects of the E457A mutant in peripheral tissues, we measured the expression of negatively and positively regulated genes in the liver. $Gsta$ was used as a negatively regulated gene in the liver (21). The $Gsta$ expression profile was similar to that of $Tshb$: mRNA levels in the TRβ(E457A/E457A) mice were not suppressed after administration of the highest L-T$_3$ dose (Figure 6A), which indicated markedly reduced sensitivity to TH inhibition of gene expression. 5’ deiodinase type I (Dia1) was used as a positively regulated gene in the liver. Dia1 mRNA levels increased in the TRβ(WT/WT) and TRβ(E457A/WT) animals after L-T$_3$ treatment, although the increase in Dia1 expression was lower in heterozygous animals. In contrast, Dia1 expression in TRβ(E457A/E457A) mice was insensitive to L-T$_3$ treatment (Figure 6B).

Discussion

Negative feedback regulation of the H-P-T axis has been well studied because of its physiological importance. A number of reports


have identified elements responsible for negative regulation and proposed mechanisms and target cofactors based on the traditional “on-DNA” hypothesis (9, 12, 22) — i.e., TR bound to DNA. Recently, one group has suggested that the conformation of the CoR bound to the TH response element (TRE) determined whether it would be an nTRE or pTRE (23). Alternatively, “off-DNA” models for negative regulation have also been proposed by several groups — i.e., TR not bound to DNA. These models depend on the ability of non–DNA-bound TR to sequester a limiting transcription factor in the absence (nuclear CoR model) or presence (activator protein 1 model) of T₃ (15, 24, 25). These models question, therefore, whether nTREs truly exist.

We mutated the DNA-binding P box domain of TR-β in mice (GS125) to critically test the validity of these models (19). Using these animals, we showed that DNA binding by TR on negatively regulated genes was essential for the T₃-mediated transcriptional regulation, which indicates that the off-DNA hypothesis is likely not valid in vivo. Of course, it is possible that the TR DNA-binding domain interacts directly with cofactors off-DNA, via a yet-to-be-described mechanism, and that the GS125 mutation blocks this interaction. Given the minimal change to the DNA-binding domain, however, this mechanism seems less likely. Thus, negative regulation by TH, like positive regulation, requires TR binding to target gene elements.

While both negative and positive regulation by TH require receptor DNA binding, there are fundamental differences in these processes. On positively regulated genes, unliganded TRs occupy pTREs and silence gene expression through association with CoRs, such as NCoR and SMRT (7). Transcription repression is mediated through the recruitment of mSin3A (isoform A of the mammalian Sin3 protein) and histone deacetylases (26), which compact nucleosomes into a tight and less accessible structure. When T₃ binds to the TR, TR homodimers are dissociated, CoRs are released, TR/retinoid X receptor (TR/RXR) heterodimeric binding predominates, and CoAs such as SRC-1 with histone acetyltransferase (HAT) activity are recruited to the TR activation function–2 (AF-2) domain (8). However, this model cannot explain negative regulation by TH, which is essentially the opposite process. In the absence of T₃, TR activates gene expression, while in the presence of T₃, expression is repressed. The fact that DNA binding is essential for negative regulation indicates 1 of the following possibilities: (a) the same cofactor acts as a coactivator or corepressor on positive versus negative TREs, respectively; or (b) different cofactors are recruited to mediate positive versus negative T₃ regulation.

The present study shows that the E457A mutation in vivo significantly affects T₃ regulation, which suggests a high degree of resistance to TH. Elevated TSH subunit mRNA levels, serum TSH concentrations, and TH levels demonstrate that regulation of the H-P-T axis is impaired in E457A mutant mice, which indicates that an intact CoA-binding surface is essential for negative feedback regulation of the H-P-T axis in vivo. This defect in negative regulation is more severe than that reported in TR-β–KO animals and activates gene expression, while in the presence of T₃, expression is repressed. The fact that DNA binding is essential for negative regulation indicates 1 of the following possibilities: (a) the same cofactor acts as a coactivator or corepressor on positive versus negative TREs, respectively; or (b) different cofactors are recruited to mediate positive versus negative T₃ regulation.

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mice according to quantitative RT-PCR analysis. Data were normalized for each mRNA level relative to values for the WT animals. Five to 10 animals were evaluated in each group, and data are shown as mean ± SEM.

The response of a tissue to TH is directly dependent on several factors, including the amount of hormone available, the conversion rate of T4 to T3, the number of TRs, and the quantity and nature of transcriptional co- and coregulators available within the cell. We controlled for differences in TH levels by rendering mice hypothyroid and then replacing T3 with escalating L-T4 doses. Also, TR receptor isoforms were not different among the animals. So differences seen between groups are directly related to the point mutation and its effect on coactivator binding. The observation that SRC-1–KO mice have a mild resistance to steroid and TH confirmed that SRC-1 functions physiologically as a CoA. Although our data demonstrate that the E457A mutation blocks SRC-1 binding, it must also interfere with other unknown factors to explain the higher resistance to TH seen in the pituitary (Figure 3 and 5) and in the liver (Figure 6) of these mice.

Ligand-dependent recruitment of CoAs would seem counterintuitive to a mechanism of negative regulation, whereby ligand reduces gene expression. Recently, however, Xu et al. (36) have shown that CoA-associated arginine methyltransferase 1 (CARM1) can methylate the P160 CoA class and reduce its HAT activity. In the E457A animals, the absence of SRC-1 binding would prevent methylation by CARM1 and might thereby be a mechanism explaining
the lack of negative regulation in these animals. Alternatively, a unique cofactor could be recruited to the liganded TR resulting in repression by T3. At least 2 cofactors have been described that could function in this way: RIP140 and ligand-dependent CoR (L-CoR) (37, 38). These cofactors are recruited to nuclear hormone receptor AF-2 domains in the presence of ligand; but in contrast to CoAs, these cofactors repress gene transcription. They do so by recruiting either histone deacetylases or a different repressing protein termed C-terminal binding protein (39, 40). Here we show that the E457A mutation in the TR-β2 prevents the recruitment of RIP140 in vitro, which could explain the inability of T3 to repress TRH and TSH-β subunit gene expression in vivo.

In summary, the AF-2 domain of TR-β is required for both positive and negative regulation by TH in vivo. Paradoxically, the AF-2 domain must also function as a ligand-dependent repressor in vivo, given the defect in negative regulation in E457A mice. Further studies will be required to determine whether ligand-dependent CoAs, with modified function, or ligand-dependent CoRs mediate this regulatory effect.

Methods

Transfections

Plasmid constructions. A point mutation of the Thrb was introduced by site-directed mutagenesis in the context of the TR-β2 isoform. The E457A mutant TR-β2 cDNA was inserted into the pSG5 expression vector at an EcoRI site. A luciferase reporter construct containing a positive TRE (DR-4 LUC) has been previously described (41). Negatively regulated gene reporters have also been described: human TRH -900 to +55 bp (TRH LUC) and human TSH-β -1192 to +37 bp (TSH LUC) (17). TK109, a minimal thymidine kinase promoter in pA3Luc, was used as a negative control (42).

Cell culture and transient transfection assay. HEK 293 T cells were maintained in DMEM supplemented with 10% FCS, 100 µg/ml streptomycin and amphotericin. Transient transfections were performed in subconfluent 6-well plates; 1.7 µg of reporter construct with 0.075 µg of receptor expression vector or the same amount of pSG5 vector alone were added to each well using the calcium phosphate technique (11, 17). After 15–18 hours, cultures medium containing FBS depleted of TH by treatment with anion exchange resin (AG 1-X, analytical grade; Bio-Rad Laboratories) was added. Twenty-four hours after transfection, 1 nM T3 was added, and 24 hours after transfection, cultures were harvested and assayed for luciferase activity.

Gel mobility shift assay. Gel mobility shift analysis of TRβ2 interactions was conducted with nuclear hormone receptor interaction domains of the CoRs N-CoR (43) and RIP140 (37) and the CoA SRC-1 (22). WT (TR-β2), mutant TR-β2 (E457A), and RXR proteins were derived from in vitro transcription/translation reactions in rabbit reticulocyte lysate (Promega). Interacting domains of the cofactors were synthesized as Gata fusion proteins. Fifty nanograms of N-CoR or 500 ng of SRC-1 and RIP140 were used in the indicated lanes. Interactions were tested on radiolabeled DR-4 element as previously described (41). In some reactions, T3 was added at a concentration of 10 nM.

Generation of E457A mutant knock-in mice. The targeting construct was electroporated into ES cells of the 129 strain, and G418 neomycin-resistant colonies were isolated and expanded. ES cells electroporated with the targeting vector were screened by Southern blot analysis with an external probe. Two ES clones of the construct were then injected into C57BL/6 blastocysts to generate chimeric mice, and chimeric mice were bred with C57BL/6 female mice. Chimeric males derived from targeted ES cells clones transmitted the mutation through crosses with C57BL/6 females, and the resulting heterozygous mice were interbred. Genotyping of tail DNA from E457A animals was performed by Southern blot analysis.

Mice were maintained under 12-hour light/12-hour dark cycles (beginning at 6 am/6 pm). All mice used in these experiments were of the same mixed background strain (129/C57BL/6), and WT animals were littermate controls. All animal experiments were performed according to the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of Chicago.

Serum TH and TSH measurements. Serum TH levels (total T4 and T3) were measured by solid-phase radioimmunoassay (Coat-A-Count; Diagnostic Products Corp.). Mouse serum TSH levels were measured by a sensitive heterologous radioimmunoassay, as described previously (44). TH deficiency was induced in 8-week-old mice in each of 3 groups – WT (TRβ2/WT), heterozygous (TRβE457A/WT), and homozygous (TRβE457A/E457A) – on a LoI/PTU diet (Harlan Teklad Co.) and 0.05% MMI in water. After 5 weeks, animals received either vehicle or daily subcutaneous injections of a low (0.2 µg/100 g body wt/d), medium (0.5 µg/100 g body wt/d), or high (1.0 µg/100 g body wt/d) dose of L-T3 (Sigma-Aldrich) for 5 days each. The LoI/PTU diet and MMI in water were given throughout the L-T3 treatment period. The animals were sacrificed 24 hours after the last injection of L-T3.

RNA analysis. Total RNA was extracted using a standard method (TRIZOL Reagent; Invitrogen Corp.). RT and PCR analyses were carried out on 1 µg of total pituitary RNA using AMV RT and TH DNA polymerase (Access RT-PCR System; Promega). For PCR analysis, 1 pair of primers, 5′-GACCAAGCGGAACCTTATAGAC-3′ (P1) and 5′-ACAACAAAGAAGCAAGAAGAACA-3′ (P2), was used to amplify the ligand-binding domain of the TR-β ( exon 4 to exon 7; Figure 2C). TR-PCR products from each genotype were analyzed by direct cycle sequencing using the following internal primers: 5′-GCAACAGCCAGAGCCCGAAG-3′ (S1) and 5′-CAGTCAGTCCTGAGGGATGC-3′ (S2).

For quantitative real-time RT-PCR analysis, RT was carried out on 2 µg of total pituitary or liver RNA. Real-time RT-PCR analyses were performed in a fluororescent temperature cycler (MiyQ Single-Color Real-Time PCR Detection System; Bio-Rad Laboratories) according to the recommendations of the manufacturer. Briefly, after initial denaturation at 95°C for 2 minutes and 95°C for 10 minutes, reactions were cycled 40 times using the following parameters for all genes studied: 95°C for 15 seconds, 60°C for 30 seconds, and 70°C for 30 seconds. SYBR Green (Bio-Rad Laboratories) fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during that cycle. Primers used for the amplification of cDNAs of interest were synthesized by Integrated DNA Technologies Inc. The sequence of the forward and reverse primers, respectively, were: 5′-GGTGATGGCCTGTGGTCTTCC-3′ and 5′-GACCTCGTAGTATCTCTCACTCTGGT-3′ for Thrb; 5′-TCCTGCGCCCTCTCTCCGGTCTC-3′ and 5′-AGTTGGTCTGAAGCCTCTGTG-3′ for Tsha; 5′-CCCTTGTAGCAGAAGAGAACGAG-3′ and 5′-GCTGGGATTACCGCAGG-3′ for Dio1; and 5′-CTGGAAGTACACATCAAGAGAA-3′ and 5′-GCTGGGATTACCGCAGG-3′ for Rn18s; 5′-GACATTGCCGGGAAGC-3′ and 5′-ACCTGATGCACTCATTGCG-3′ for Gata; 5′-AGCCACGCCTCTACGGGGC-3′ and 5′-CCCTTGATACAGACATCGTCCG-3′ for Dpad; 5′-TTTCTCTCCCTCCCT CCTTTT-3′ and 5′-GGCTGGAGGGTCTGAGGGG-3′ for Thra; and 5′-CAGGCGGGGAGCAACAGGAAGA-3′ and 5′-TTCAGACATCTTACAGCTC-3′ for Thrb.

We determined relative mRNA levels (2ΔCt) by comparing the PCR cycle threshold (Ct) between groups. We checked the purity of the PCR products by analyzing the melting curves. Each sample was measured in duplicate, and each experiment was repeated at least 3 times. Results are expressed relative to the values for WT expression, which were considered to be equal to 100%.

Histology. Thyroid glands were fixed and stained with hematoxylin and eosin, and the architecture was analyzed as described previously (45).

Statistical analysis. Data are reported as mean ± SEM. One-way ANOVA followed by Student-Newman-Keuls multiple comparisons test was employed for assessment of significance when comparisons were made.
within the same genotype. Two-way ANOVA was employed when mice of different genotypes and treatment were compared (GraphPad Prism version 4.0a; GraphPad Software Inc.). Differences were considered to be significant at P < 0.05.

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