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ACCELERATED COMMUNICATION

Negative Regulation of Superoxide Dismutase-1 Promoter by Thyroid Hormone

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ABSTRACT

The role of thyroid hormone [L-3,5,3′-triiodothyronine (T_3)] and the thyroid hormone receptor (TR) in regulating growth, development, and metabolic homeostasis is well established. It is also emerging that T_3 is associated with oxidative stress through the regulation of the activity of superoxide dismutase-1 (SOD-1), a key enzyme in the metabolism of oxygen free radicals. We found that T_3 reverses the activation of the SOD-1 promoter caused by the free radical generators paraquat and phorbol 12-myristate 13-acetate through the direct repression of the SOD-1 promoter by liganded TR. Conversely, the SOD-1 promoter is significantly stimulated by unliganded TRs. This regulation requires the DNA-binding domain of the TR, which is recruited to an inhibitory element between −157 and +17 of the SOD-1 promoter. TR mutations, which abolish recruitment of coactivator proteins, block repression of the SOD-1 promoter. Conversely, a mutation that inhibits corepressor binding to the TR prevents activation. Together, our findings suggest a mechanism of negative regulation in which TR binds to the SOD-1 promoter but coactivator and corepressor binding surfaces have an inverted function. This effect may be important in T_3 induction of oxidative stress in thyroid hormone excess.

Thyroid hormones control growth, development, and metabolism in virtually all mammalian tissues. Indeed, a primary role of L-3,5,3′-triiodothyronine (T_3) is to regulate oxygen consumption and metabolic rate (Yen, 2001; Baxter and Webb, 2006). More recently it has emerged that thyroid hormones are associated with the induction of oxidative stress in certain tissues. In fact, the hypermetabolic state in hyperthyroidism is associated with oxidative tissue injury, including alterations of heart electrical activity, muscle weakness, and liver injury (Venditti and Meo, 2006).

Oxidative injury is normally limited through the activity of the superoxide dismutase (SOD) enzymes, which serve as the first line of defense against the damaging effects of superoxide radicals (O_2^-) by convert O_2^- to hydrogen peroxide. Of the

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ABBREVIATIONS: T_3, L-3,5,3′-triiodothyronine; SOD, superoxide dismutase; TR, thyroid hormone receptor; TRE, thyroid hormone response element; GRIP, glucocorticoid receptor-interacting protein; SRC-1, steroid receptor coactivator-1; PMA, phorbol 12-myristate 13-acetate; GST, glutathione *S*-transferase; HTC, hepatoma tissue culture; DTT, dithiothreitol; SMRT, silencing mediator for retinoid and thyroid receptors; TST, Tris/saline/Tween 20; TRH, thyrotropin-releasing hormone; TSH, pituitary thyroid-stimulating hormone; DBD, DNA binding domain; wt, wild-type; RTH, resistance to thyroid hormone; F451X, deletion of helix 12; G345R, mutation in the ligand binding domain; GS125, mutation in the DBD; I280K, mutation in the corepressor binding site; GAL-4 TRβ1, chimerical TR consisting of the TRβ1LBD fused to GAL-4 DBD.

different SOD enzymes, SOD-1 is the most abundant (90%) and is widely distributed (Johnson and Giulivi, 2005). *Drosophila melanogaster* that lack SOD-1 shows a reduced life span (Phillips et al., 1989). Moreover, perturbations in SOD-1 activity have been associated with several diseases (Peled-Kamar et al., 1995; Stathopulos et al., 2003).

The antioxidant defense system is influenced by the thyroid hormone status. For example, thyroxine treatment decreases Cu/Zn SOD (SOD-1) activity in the liver of old rats (Saicic et al., 2006). Conversely, progressive hypothyroidism leads to an increase of superoxide dismutase activity in the brain of rats (Rahaman et al., 2001). cDNA microarray experiments to identify genes perturbed in hyperthyroid rat hearts revealed a number of genes, including SOD-1, that were down-regulated by T_3 (De et al., 2004). Although the inverse association between SOD-1 and T_3 in several tissues was clear, the mechanism involved in this regulation remained poorly understood.

The genomic actions of thyroid hormone are mediated by TRs, which are ligand-regulated transcription factors belonging to the nuclear receptor superfamily (McKenna and O'Malley, 2002; Nettles and Greene, 2005). The molecular mechanism of positive transcriptional regulation by TR is well established. TRs interact directly with specific DNA sequences, known as thyroid hormone response elements (TREs) (Yen et al., 2006). Unliganded TRs recruit specific corepressor proteins that, in turn, form part of a large corepressor complex that contains histone deacetylases and represses transcription of nearby genes by condensing chromatin (Li et al., 2000; McKenna and O'Malley, 2002; Codina et al., 2005). Ligand binding induces changes in receptor conformation and dynamics (Nagy and Schwabe, 2004) that lead to the release of corepressors and subsequent recruitment of p160 coactivators, such as glucocorticoid receptor-interacting protein (GRIP1) and steroid receptor coactivator-1 (SRC-1) (Ribeiro et al., 1998).

In contrast to positive regulation, the molecular mechanism of negative regulation by nuclear receptors is less well understood. Several hypotheses have been proposed to explain the action of TR on negative TREs (Lazar, 2003). One hypothesis is that the TR directly regulates transcription through direct binding to target promoters, either to unusual DNA response elements or via protein-protein interactions with other transcription factors associated with cognate response elements. Another hypothesis suggests that the role of TR is indirect through the squelching of coregulators from other transcription factors.

In this study, we sought to understand the mechanism through which T_3 regulates the proximal region of the SOD-1 gene promoter. We showed that T_3 could reverse the activation of the SOD-1 promoter caused by free radical generators, such as paraquat and PMA. We observed that TRβ1 (and also TRα1) activates SOD-1 promoter in the absence of ligand, and T_3 reversed this activation in a dose-dependent manner. We found that the region of the SOD-1 promoter between -157 and the $+17$ was essential for TRβ1 regulation, and this regulation requires the TR DNA binding domain for binding to the proximal region of the SOD-1 promoter. TR mutants that were defective in corepressor recruitment no longer activated the SOD-1 promoter. Conversely, a receptor that was defective in coactivator recruitment, but was still able to interact with corepressor, showed impaired down-regulation in response to T_3. We therefore suggest that TR may play a role in oxidative stress by directly binding to the SOD-1 promoter, but TR coregulator binding surfaces have an inverted function. This effect may be important in production of intracellular superoxide radicals in conditions of thyroid hormone excess.

Materials and Methods

Plasmids. The TR mutants F451X, G345R, and GS125 TRβ1 were created with the use of QuikChange site-directed mutagenesis kits (Stratagene, La Jolla, CA) into the pCMX vector that encodes 461 amino acids of hTRβ1 sequence. The mutated sequence was verified by DNA sequencing using Sequenase kits (Stratagene). The five deletions of SOD-1 promoter cloned upstream of the luciferase gene (Minc et al., 1999) were kindly provided by Dr. Christian Jaulin [Centre de Recherche en Cancérologie (E229), Montpellier, France]. Plasmids encoding hTRβ1 (Ribeiro et al., 2001) Gal-4 hTRβ1, GAL-responsive element-5 Luciferase, GST-GRIP1 (563–767) (Darimont et al., 1998), GST-SRC1a (381–882) (Feng et al., 1998), GST-SMRT (987–1491) (Webb et al., 2003), and TR mutant I280K (Marimuthu et al., 2002) were gifts from Dr. J. D. Baxter (University of California, San Francisco, CA).

Cell Culture and Transfection. U937, MG63, and rat hepatoma tissue culture (HTC) cells were maintained and subcultured in RPMI-1640 medium or Dulbecco's modified Eagle medium, containing 5% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, at 37°C and 5% CO_2. Transfection procedures were described previously (Ribeiro et al., 2001), with some modifications. In brief, MG63 and HTC cells were divided 48h before transfection to generate 40 to 60% confluence in 150-mm plates at the time of transfection. Cells were collected by centrifugation and then resuspended in transfection solution (1.5×10^7 cells/0.5 ml) containing DMEM without phenol red (Invitrogen, Carlsbad, CA) and 250 mM sucrose, and then cotransfected with 3 μg of SOD-1 luciferase reporter gene, 500 ng of control β-galactosidase vector and 1.5 to 4.5 μg of wtTRβ1 expression vector or its mutants. Cells were transferred to a cuvette and then electroporated by using a Bio-Rad gene pulser under 290 mV and 960 μF. After electroporation, cells were transferred to fresh media and then plated in 12-well multiplates and treated with T_3 (10^{-7} M or different concentrations) or ethanol (control). After 24 h, cells were collected by centrifugation, lysed by the addition of 150 μl of 1× lysis buffer (Promega), and assayed for luciferase and β-galactosidase activity (kit from Promega Corp.). Transfection data are mean ± S.E.M. of a minimum of triplicate samples that were repeated three to five times. The empty vector pCMX was used as a control for the transfections without TR (Fig. 1B). Because we noticed no difference between transfections with SOD-1 promoter alone and cotransfections with empty pCMX vector (data not shown), some assays were performed in absence of pCMX.

Gel Shift Assay. Binding of TR to DNA was assayed by mixing 20 fmol of ^{35}S-labeled TRβ1 or GS125 TRβ1 produced in a reticulocyte lysate system, TNT T7 (Promega, Madison, WI), in the presence or absence of 10^{-6} M T_3, with 600 fmol of unlabeled different SOD-1, DR-4 (5′-AGTTC AGGTCA CAGG AGGTCA GAG-3′) and inverted palindrome F2 (5′-TTC TGACCC CATTGG AGGTCA-3′) oligonucleotides, and 1 μg of poly(dI-dC) (GE Healthcare, Little Chalfont, Buckinghamshire, UK) in a 20-μl reaction mixture. The binding buffer contained 25 mM HEPES, 50 mM KCl, 1 mM DTT, 10 μM $ZnSO_4$, 0.1% Nonidet P-40, and 5% glycerol. After 30 min at room temperature, the mixture was loaded onto a 5% nondenaturing polyacrylamide gel that was previously run for 30 min at 200 V. To visualize the TR-DNA complexes, the gel was run at 4°C for 120 min at 240 V, in a running buffer containing 6.7 mM Tris, pH 7.5, 1 mM EDTA, and 3.3 mM sodium acetate. The gel was then fixed, treated with Amplify (GE Healthcare), dried and exposed for autoradiography. TRs used in this assay were quantified through [^{125}I]T_3 binding

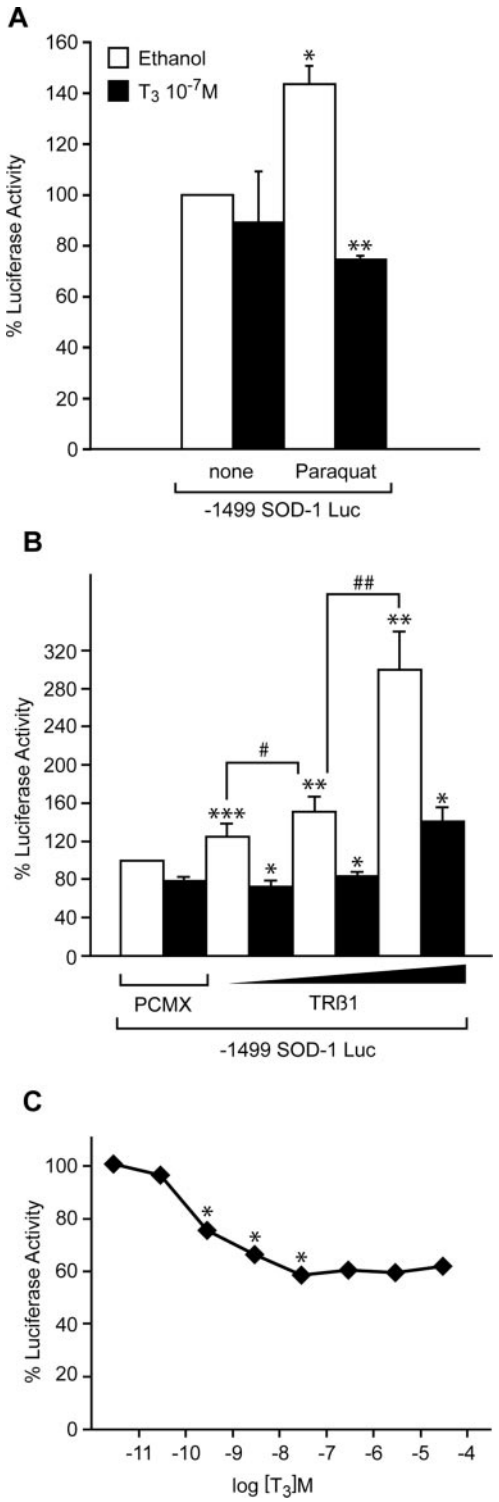

Fig. 1. Regulation of SOD-1 by thyroid hormone receptor. A, human promonocytic U937 cells were cotransfected with 3 μg of a reporter gene containing a construction pGLS −1499 SOD-1 promoter encoding luciferase (SOD-1 Luc) and treated or not with 50 μM paraquat; *, $P < 0.001$ versus no TR; **, $P < 0.001$ versus no T_3. B, U937 cells were cotransfected with 1.5, 3.0, and 4.5 μg of expression vector encoding wt hTRβ1 and 3 μg of −1499 SOD-1 Luc; *, $P < 0.001$ versus TR no T_3; **, $P < 0.001$ versus no TR/no T_3; ***, $P < 0.01$ versus no TR/no T_3; #, $P < 0.01$; ##, $P < 0.001$. C, U937 cells were cotransfected with 1.5 μg of wt hTRβ1 and 3 μg of −1499 SOD-1 Luc and then treated with increasing amounts of T_3; *, $P < 0.001$ versus no T_3. Luciferase activity was expressed as percentage of −1499 SOD-1 Luc in the absence of T_3 and without or with cotransfected wt hTRβ1.

assay. Amounts used for gel shift assay were also confirmed through SDS-PAGE run of ^{35}S-labeled TRs, where gels were fixed, dried, and exposed for autoradiography. Bands visualized in X-ray films were quantified with a Kodak imager (Eastman Kodak, Rochesteer, NY). SOD-1 oligonucleotides (Fig. 4A): Seq1 (−87 to −46), GAGCGCGTGCGAGGCGATTGGTTTGGGGCCAGAGTGGGCGAG; Seq1mut (in bold; −87 to −46), GAGCGCGTGCGAGGCGATTGG**ATGCAT**GCCAGAGTGGGCGAG; Seq 2 (−51 to −7), GGCGAGGCGCGGAGGTCTGGCCTATAAAGTAGTCGCGGAGACGGG; Seq 3 (−12 to +29), GACGGGGTGCTGGTTTGCGTCGTAGTCTCCTGCAGCGTCTGG; Seq 4 (+23 to +69), TCTGGGGTTTCCGTTGCAGTCCTCGGAACCAGGACCTCGGCGTG; and Seq 5 (+64 to +104), GGCGTGGCCTAGCGAGTTATGGCGACGAAGGCCGTGTGCG.

GST Pull-Down Assay. pCMX-TRβ1wt or pCMX-mutants vectors were used to produce radiolabeled full-length receptor in vitro, using the TNT-Coupled Reticulocyte Lysate System (Promega) and [^{35}S]methionine. GST SRC1a (381–882), GST-GRIP1 (563–767), and GST-SMRT (987–1491) fusion proteins were prepared using conventional protocols (Pfizer, New York, NY). In brief, the plasmids were transformed into BL21, cultured into 2xLB medium, pelleted and resuspended in 1× TST buffer (50 mM Tris pH 7.5, 150 mM NaCl, and 0.05% Tween 20) with 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail 1:1000 (Sigma, St. Louis, MO). Then, the solution was incubated with lysozyme and sonicated (three 2.5-min cycles, amplitude 70%, 1 pulse/s with a break of 5 min between each cycle).

The debris were pelleted and the supernatant was incubated for 2 h with 500 μl of glutathione-Sepharose 4B beads equilibrated with 1× TST. GST fusion protein beads were washed with with 1× TST containing 0.05% Nonidet P-40 and resuspended in 1× TST with 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail 1:1000 (Sigma), and 50% glycerol, and then stored at −20°C. All procedures above were carried out at 4°C. For the binding assay, the glutathione bead suspension containing 4 μg of GST fusion protein was incubated with 3 μl of ^{35}S-labeled protein in 150 μl of 1× TST buffer with 0.1% Nonidet P-40, 0.1% Triton X-100, 1 mM DTT, and 2 μg/ml bovine serum albumin, in the presence of 10^{-6} M T_3 or vehicle. After a 2-h incubation at 4°C, the beads were washed with the same incubation buffer. The beads with associated proteins were analyzed on 10% SDS-polyacrylamide gels and visualized by autoradiography.

Statistical Analysis. One-way analysis of variance followed by Student-Newman-Keuls multiple comparison test was employed for assessment of significance (Prism version 4.0a; GraphPad Software Inc., San Diego, CA). Differences were considered to be significant at $P < 0.05$.

Results and Discussion

The SOD-1 Promoter Is Negatively Regulated by TRβ1/T_3. To explore the regulation of the proximal SOD-1 gene, we used a reporter plasmid with the proximal promoter region −1499 to +17 of the SOD-1 fused to the luciferase gene (SOD-luc). Here, we observe that treatment with T_3, the active thyroid hormone, reversed the effect of the paraquat through a direct or indirect repression of the SOD-1 promoter activity (Fig. 1A).

To determine whether the effect of T_3 on the SOD-1 promoter was mediated by the TR, we examined the effect of transfected TRβ1 on the SOD-1 promoter activity in U937 cells (Fig. 1, B and C), human osteosarcoma MG63 cells (data not shown) and rat HTC cells (Fig. 3B) in the presence or absence of T_3. We observed that unliganded TRβ1, also TRα1 (data not shown), activated the SOD-1 promoter and that T_3 reversed this effect. TRβ1 activated the SOD-1 promoter in U937 cells by 2- to 3-fold in a concentration-dependent man-

ner, and T_3 treatment reversed this activation by 50 to 60% (Fig. 1B). T_3 repression was dose-dependent (Fig. 1C), with maximum inhibitory effect at 0.5 nM, typical for thyroid hormone responses.

The SOD-1 promoter behaved similarly to those of thyrotropin-releasing hormone (TRH) (Feng et al., 1994) and the pituitary thyroid-stimulating hormone (TSH) α- and β- subunit genes (Chatterjee et al., 1989; Bodenner et al., 1991), which all contain nTREs.

A T_3-Responsive Sequence in SOD-1 Promoter. To characterize the element responsible for unliganded TRβ1 activation and T_3 inhibition in the SOD-1 proximal promoter, we examined effects of T_3 upon different 5′ deletions of the SOD-1 promoter. Constructs with a 5′ boundary of pGLS −157 or longer were repressed by T_3 (Fig. 2A). The shorter construct pGLS −71/+17 also showed a significant response to T_3 treatment but the constitutive activity of the promoter was so much lower that one could not be fully confident that the full T_3 response was preserved. Together, these results suggest that a T_3 response element is located in the nucleotide −157 to +17 region.

To confirm the opposing activities of free radical generator and T_3 on the −157/+17 SOD-1 promoter, we tested the effect of PMA on SOD-1 Luc construct cotransfected with TRβ1. As expected, PMA activated this promoter and T_3 antagonized this effect (Fig. 2B).

The DBD of TR Was Required to Regulate the SOD-1 Promoter. To understand whether the regulatory effect of the TR on the activity of the SOD-1 promoter required TRβ1 DNA-binding domain, we examined the activity of a chimeric TR lacking the DNA binding domain (DBD) but fused to the heterologous GAL-4 DBD (GAL-4 TRβ1) (Fig. 3A). This chimeric protein showed a lower activation of the SOD-1 pro-

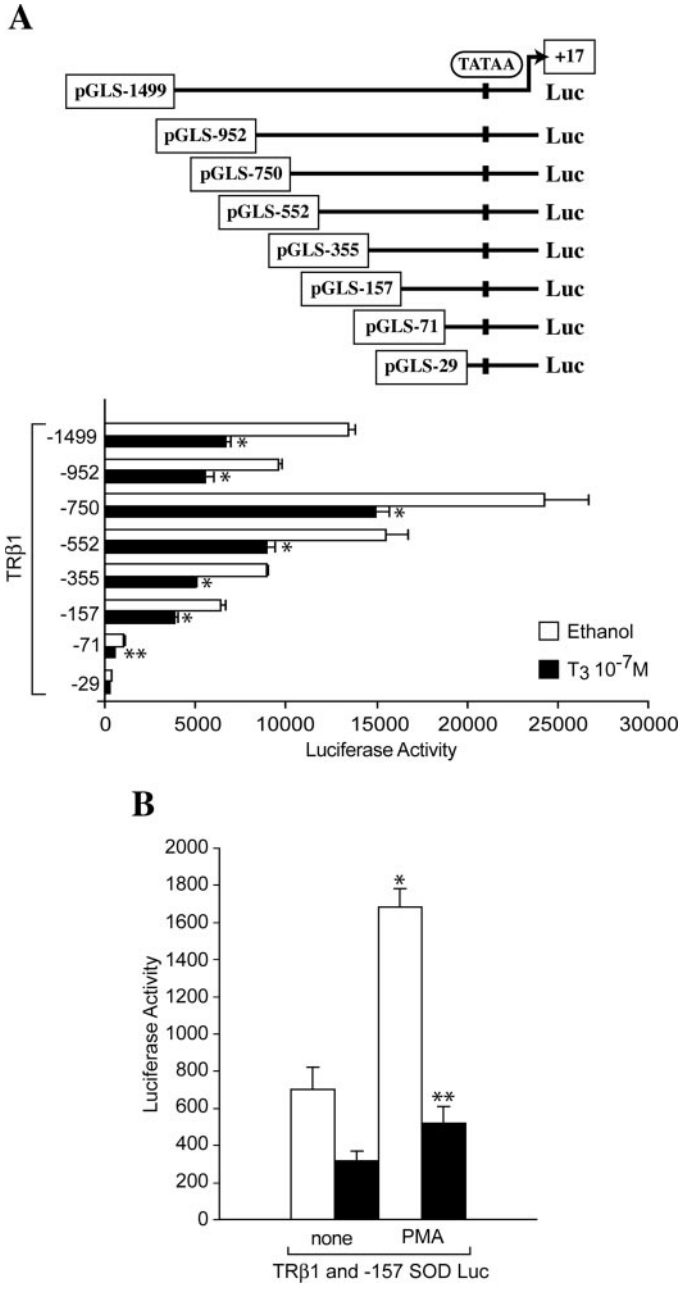

Fig. 2. TRβ1 activation and T_3 inhibition in different 5′ deletions of the SOD-1 promoter linked to the luciferase gene. U937 cells were cotransfected with 1.5 μg of expression vector encoding hTRβ1wt and 3 μg of different pGLS constructs of SOD-1 promoter encoding luciferase; *, $P <$ 0.001 versus no T_3; **, $P < 0.05$ versus no T_3 (A) or with −157 SOD1 Luc, treated or not with 100 ng/ml PMA; *, $P < 0.001$ versus no PMA/no T_3; **, $P < 0.001$ versus PMA/no T_3 (B). The data show a representative experiment, which was repeated 3–4 times.

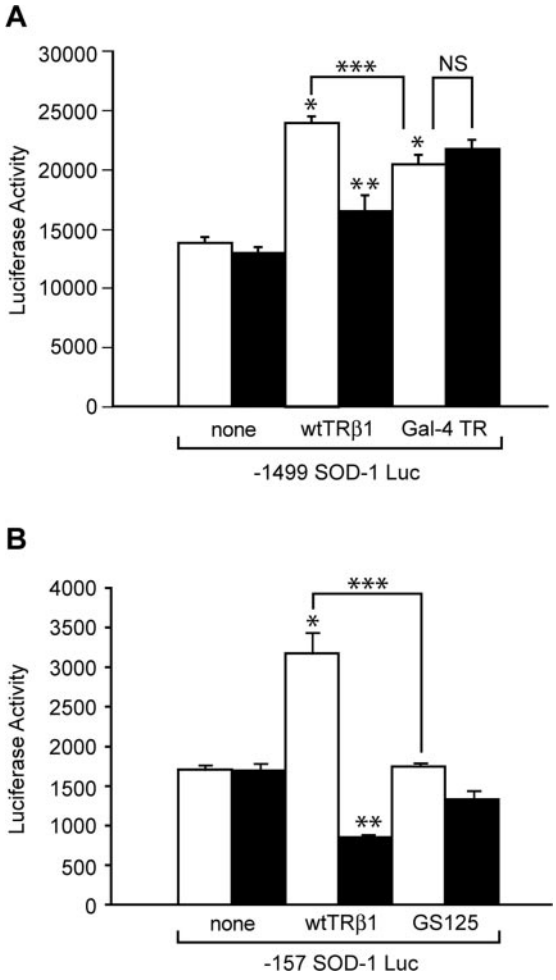

Fig. 3. DBD is required to regulate SOD-1 promoter. A, U937 cells were cotransfected with 3 μg of −1499 SOD-1 Luc and 1.5 μg of GAL-4 TRβ1 or wt hTRβ1; *, $P < 0.001$ versus no TR/no T_3; **, $P < 0.001$ versus TR/no T_3; ***, $P < 0.001$. B, HTC cells were cotransfected with 3 μg of pGLS −157 SOD-1 Luc and 1.5 μg of TRβ1 or GS125 hTRβ1; *, $P < 0.001$ versus no TR/no T_3; **, $P < 0.001$ versus TR/no T_3; ***, $P < 0.001$. The data show a representative experiment that was repeated three or four times.

moter compared with wtTR and did not repress SOD-1 promoter in the presence of T_3. GAL-4 TRβ1 did activate GAL luciferase reporter gene in presence of T_3 (data not shown), indicating that this protein was functionally active. In addition, we prepared and analyzed the activity of a TRβ1 mutant, GS125, as described previously for TRβ2 (Shibusawa et al., 2003b). This mutant did not regulate the −157 SOD-1 promoter (Fig. 3B) but showed the same binding affinity to T_3 as wt TRβ1/T_3, confirming that it was functional (data not shown).

Nuclear receptors regulate transcription by binding to specific DNA sequences in target genes but can also modulate gene expression by mechanisms independent of DNA binding. Analysis of the "knock-in" mouse that harbors a TRβ mutant defective in DNA binding described by Shibusawa et al. (2003a) revealed that thyroid hormone failed to suppress TSH gene transcription in these mice, supporting the conclusion that negative regulation of the TSH gene required DNA binding by TR. Our data indicate that two TR mutants that cannot bind to canonical TREs, GAL-4 TRβ1 and GS125 TRβ1, both failed to repress SOD-1 promoter activity. The GS125 TRβ2 mutant, which binds to a TRE/glucocorticoid response element promoter but showed low affinity for positive and negative TREs, abolishes transactivation on three classic pTREs (DR4, LAP, and PAL) and all negatively regulated promoters in the hypothalamic-pituitary-thyroid axis (TRH, TSHβ, and TSHα) (Shibusawa et al., 2003b). Thus, our results suggest that TR DNA binding activity is required for regulation of the SOD-1 promoter.

TRβ1 Bound to SOD-1 Promoter. To test the hypothesis that TR binds to the SOD-1 promoter, we performed gel-shift assays with radiolabeled TRβ1 and different sequences from

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Seq 1     (-87 to -46):   GAGCGCGTGCGAGGCGATTGGTTTGGGGCCAGAGTGGGCGAG
Seq 1mut  (-87 to -46):   GAGCGCGTGCGAGGCGATTGGATGCATGCCAGAGTGGGCGAG
Seq 2     (-51 to -7):    GGCGAGGCGCGGAGGTCTGGCCTATAAAGT GTCGCGGAGACGGG
Seq 3     (-12 to + 29):  GACGGGGTGCTGGTTTGCGTCGTAGTCTCCTGCAGCGTCTGG
Seq 4     (+23 to + 69):  GTCTGGGGTTTCCGTTGCAGTCCTCGGAACCAGGACCTCGGCGTG
Seq 5     (+64 to +104):  GGCGTGGCCTAGCGAGTTATGGCGACGAAGGCCGTGTGCG
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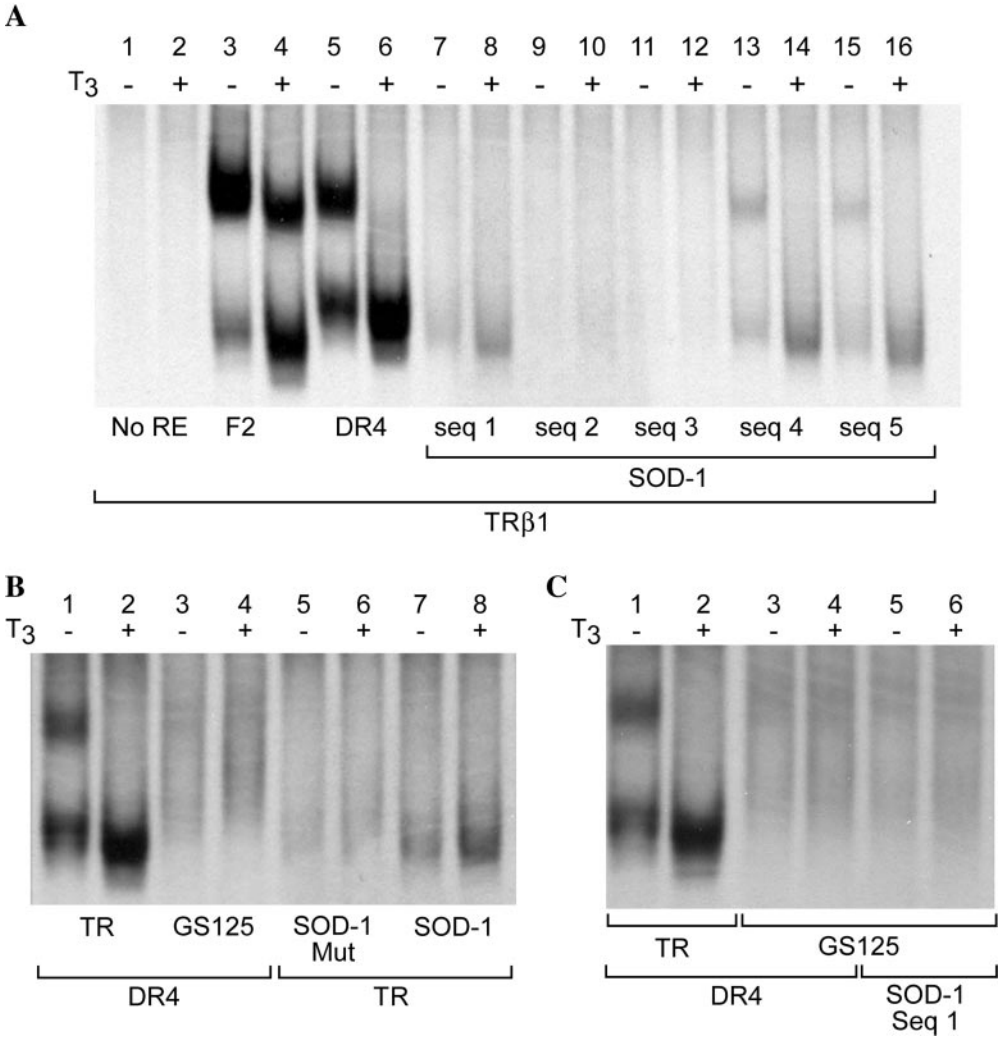
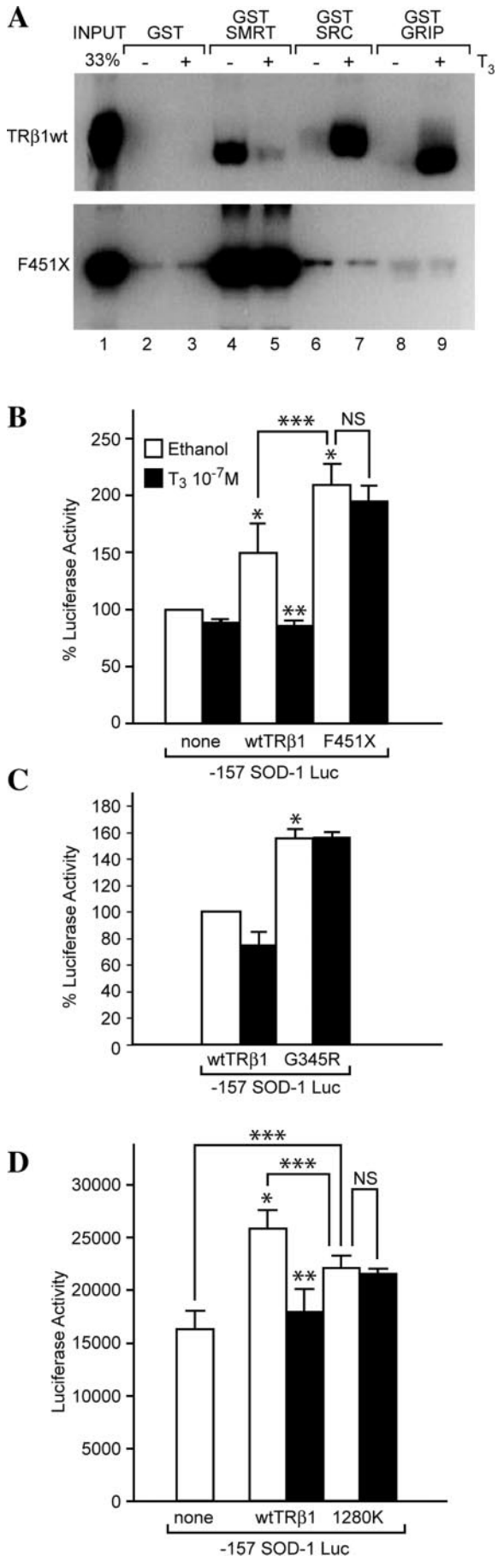


Fig. 4. TRβ1 binds to different sequence of SOD-1 promoter. Gel-shift assays contained 20 fmol of the in vitro-translated ^{35}S-labeled hTRβ1 (A–C), ^{35}S-labeled GS125 TR mutant (B and C) and 600 fmol of DR4 (A, lanes 5 and 6; B, lanes 1–4; C, lanes 1 and 2), F2 (A, lanes 3 and 4), different sequences of SOD-1 (A, lanes 7–16), or only the sequence 1 of SOD-1, mutated (B, lanes 5 and 6) or not mutated (B, lanes 7 and 8; C, lanes 5–6).



the SOD-1 promoter and the first exon of SOD-1 gene (Fig. 4A). As expected, the TRβ1 bound as a homodimer to two canonical positive TREs (F2 and DR4) in the absence of ligand, and T_3 shifted the balance toward monomer binding (Fig. 4A, lanes 3–6). Three regions of the SOD-1 sequence supported weak TRβ1 binding. The sequence 1 of SOD-1 promoter (−87 to −46) binds monomeric TR, and this binding was slightly increased in the presence of T_3 (Fig. 4A, lanes 7 and 8). Sequences from the first exon of SOD-1 transcript (+23 to +69 and +64 to +104) support weak homodimer and monomer binding (Fig. 4A, lanes 13–16); T_3 favored TR monomer formation (Fig. 4A, lanes 14 and 16). Interestingly we noticed that TRβ1 bound rather weakly to the SOD-1 promoter compared with DR4 or F2 elements.

The sequence −87 to −46 of SOD-1 promoter is close to the TATA box region and contains the sequence TTTGGG, which is also present in other negatively regulated genes characterized previously (Kim et al., 2005). Mutation of this sequence (ATGCAT) abolished TR monomer binding (Fig. 4B, lanes 5–8). Moreover, the GS125 TRβ1 mutant, which cannot regulate SOD-1 activity, was also unable to bind to the DR-4 element or SOD-1 sequences (Fig. 4, B, lanes 3 and 4, and C). Both lines of evidence suggest that TR regulated SOD-1 activity by binding to the sequence 1 negative TRE.

These results are in accordance with other studies, which showed the presence of nTREs in the promoters very close to the TATA box (Belandia et al., 1998; Perez-Juste et al., 2000). It is noteworthy that TR also binds weakly to two different sequences in the first exon of SOD-1 gene; here, unliganded TR bound as homodimers and liganded TR as monomer units. Belandia et al. proposed that T_3 represses β-amyloid precursor protein promoter activity by a mechanism that requires binding of TR to a specific sequence located in the first exon (Belandia et al., 1998).

The TR-DNA interaction observed in our study is weak compared with other positive TREs, F2 and DR4. Nevertheless, nTREs are generally composed of weak TR binding sites. Kim et al. (2005) demonstrated that nuclear receptor corepressor activates CD44 promoter by a weak unliganded TR-DNA interaction, 100-fold less than DR4. This weak TR-DNA binding was essential for CD44 regulation by T_3. Our results are in agreement with this finding, in that they showed a weak TR-SOD-1 promoter interaction.

Although our data indicate that TR monomer units were important for the repression mechanism of SOD-1 promoter by T_3, we cannot exclude the idea that squelching of coregulators might have played a part in this regulation. Our results reveal that GAL-4 TR did activate the SOD-1 promoter in the absence of hormone to a significant degree. Because the ligand binding domain of Gal-4 TR can bind to coregula-

Fig. 5. TRβ1 mutations in coactivator and corepressor binding surfaces. A, pull-down experiments examining the binding of labeled receptors to SMRT, GRIP, and SRC protein fragment. Binding is expressed as the percentage of input labeled receptor. Binding of ^{35}S-labeled wt hTRβ1 or F451X to GST-SMRT, GST-SRC, and GST-GRIP in presence or absence of 10^{-6} M T_3. U937 cells were cotransfected with 3 μg of pGLS −157 SOD-1 Luc and 1.5 μg of wt hTRβ1 or F451X (B), *, $P < 0.001$ versus no TR/no T_3; **, $P < 0.001$ versus TR/no T_3; ***, $P < 0.001$; G345R (C), *, $P < 0.001$ versus TR/no T_3; or I280K (D), *, $P < 0.001$ versus no TR; **, $P < 0.001$ versus TR/no T_3; ***, $P < 0.01$. B and C, luciferase activity was expressed as percentage of −157 SOD-1 Luc in the absence of T_3 and without (B) or with (C) cotransfected wt hTRβ1. D, the data show a representative experiment that was repeated three to five times.

tors, it is possible that the squelching mechanism could contribute to activation by unliganded TRs. Furthermore, indirect regulation through other transcription factors may cooperate with liganded TR to negatively regulate the SOD-1 promoter, because the −157 to +17 region in this promoter shows binding sites for the transcription factors: simian virus 40 promoter factor 1, activator protein-1, early growth response protein, nuclear factor-κB, and aryl hydrocarbon receptor. Of these, it has been well established that AP-1 can be subject to "trans-repression" by nuclear receptors. Therefore, we tested whether a mutation to the AP-1 site might reduce the activity of TR on this promoter (data not shown). Our results clearly showed that this was not the case.

Activation of the SOD-1 Promoter by Unliganded TR Requires the Corepressor Binding Surface. To explore the role of TR coregulator binding surfaces in SOD-1 promoter regulation, we made use of mutations that have been characterized previously. We first confirmed that a natural mutation of the TR (F451X) in which helix 12 is absent, from patients with resistance to thyroid hormone (RTH), increases TR binding to corepressor nuclear receptor corepressor (Marimuthu et al., 2002) in GST pull-down assays. In this study, we showed that T_3 decreases the binding of TR to the corepressor SMRT (Fig. 5A, wtTR lane 5) and increases binding to the coactivators GRIP and SRC (Fig. 5A, wtTR lanes 7 and 9). Furthermore, F451X shows an enhanced constitutive binding to SMRT (Fig. 5A, F451X lanes 4 and 5) and decreased binding to both coactivators (Fig. 5A, F451X lanes 6–9). In transfection assays, F451X increased Luc expression from the SOD-1 promoter by 2.2-fold and T_3 could not reverse this activation (Fig. 5B).

We also analyzed the actions of another RTH mutant (G345R), which binds corepressor (Liu et al., 1998) but cannot bind ligand (Yen et al., 1995; Takeshita et al., 1996), on SOD-1 promoter activity. Like F451X, G345R activated the −157 SOD-1 promoter but failed to repress the SOD-1 promoter in presence of T_3 (Fig. 5C). Together, these results indicate that two TRβ1 mutants that bind corepressors but not coactivators can enhance SOD-1 promoter activity.

Previous studies indicate that corepressors may be involved in activation of genes negatively regulated by thyroid hormone, such as TSHβ, TSHα, and TRH (Tagami et al., 1999; Berghagen et al., 2002). Our results are in agreement with these findings. The natural mutant F451X, where the helix 12 of wtTR was deleted and the corepressor-binding surface was exposed, enhances corepressor and inhibits coactivator binding and stimulates the SOD-1 promoter more strongly than wtTRβ1, and T_3 did not reverse this effect. Likewise, another RTH mutant that binds corepressor but not ligand or coactivator activated the SOD-1 promoter better than wtTRβ1 and, as expected, failed to repress transcription in response to T_3.

To investigate the role of the corepressor binding surface in SOD-1 promoter regulation, we used a previously characterized TR mutant that inhibit corepressor binding (Marimuthu et al., 2002). One of the residues that forms the corepressor-binding surface, Ile280, lies mostly underneath helix 12 and is solvent-inaccessible in the liganded TR-ligand binding domain structure. The mutant I280K (G. B. Barra, L. F. Ribeiro-Velasco, R. Pessanha, I. C. Ribeiro, L. A. Simeoni, R. C. J. Ribeiro, F. A. R. Nerves, manuscript in preparation) showed a decreased SMRT binding and also a weak binding to GRIP and SRC in presence of T_3. The unliganded TR I280K mutant activated neither the SOD-1 promoter nor wtTRβ1 in transfections (Fig. 5D) and did not repress SOD-1 promoter activity in the presence of T_3. Together, our data indicate that the corepressor binding surface was required for activation of the SOD-1 promoter by unliganded TRs and that the coactivator binding surface was required for T_3-dependent repression. These results indicate that the role of TR corepressor and coactivator binding surfaces was reversed at the SOD-1 promoter.

It is presently believed that nuclear hormone receptors promote dynamic recruitment of different coregulator complexes to target promoters and that these effects are associated with an equally dynamic binding of the nuclear receptor itself to the promoter (Perissi and Rosenfeld, 2005). In this context, further studies will be important to elucidate the dynamic mechanism of the recruitment of multiple complexes, such as histone deacetylases/corepressors/TR, to alter the chromatin structure surrounding the promoter of *SOD-1* gene. Nevertheless, our data support the hypothesis of an inverted role of coregulators on negative TREs.

In conclusion, we have revealed the SOD-1 promoter as a novel target for TR action. Given that SOD-1 is a key enzyme against the damaging effects of superoxide radicals, this closely associates the thyroid hormone and the formation of oxygen radicals and other reactive species, which lead to oxidative stress. In addition, this study highlights the SOD-1 promoter as a useful tool for studying genes that are negatively regulated by thyroid hormone, providing new insights into the negative regulation by nuclear hormone receptors.

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