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Nuclear Corepressors Mediate the Repression of Phospholipase A_2 Group IIa Gene Transcription by Thyroid Hormone*

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Background: Secretory phospholipase A_2 (PLA2g2a) gene expression is stimulated by cytokines and inhibited by steroid hormones.
Results: Thyroid hormone (T_3) inhibits PLA2g2a transcription via binding of the T_3 receptor (TRβ) to a negative T_3 response element.
Conclusion: Liganded TRβ recruits nuclear corepressors to the PLA2g2a promoter.
Significance: T_3 regulates PLA2g2a expression in the liver and utilizes a novel mechanism of corepressor recruitment.

Secretory phospholipase A_2 group IIa (PLA2g2a) is associated with inflammation, hyperlipidemia, and atherogenesis. Transcription of the PLA2g2a gene is induced by multiple cytokines. Here, we report the surprising observation that thyroid hormone (T_3) inhibited PLA2g2a gene expression in human and rat hepatocytes as well as in rat liver. Moreover, T_3 reduced the cytokine-mediated induction of PLA2g2a, suggesting that the thyroid status may modulate aspects of the inflammatory response. In an effort to dissect the mechanism of repression by T_3, we cloned the PLA2g2a gene and identified a negative T_3 response element in the promoter. This T_3 receptor (TRβ)-binding site differed considerably from consensus T_3 stimulatory elements. Using *in vitro* and *in vivo* binding assays, we found that TRβ bound directly to the PLA2g2a promoter as a heterodimer with the retinoid X receptor. Knockdown of nuclear corepressor or silencing mediator for retinoid and thyroid receptors by siRNA blocked the T_3 inhibition of PLA2g2a. Using chromatin immunoprecipitation assays, we showed that nuclear corepressor and silencing mediator for retinoid and thyroid receptors were associated with the PLA2g2a gene in the presence of T_3. In contrast with the established role of T_3 to promote coactivator association with TRβ, our experiments demonstrate a novel inverse recruitment mechanism in which liganded TRβ recruits corepressors to inhibit PLA2g2a expression.

Phospholipase A_2 (PLA_2)[2] is an esterase that hydrolyzes membrane phospholipids at the sn2 position to generate free fatty acids like arachidonic acid and lysophospholipids (1). Arachidonic acid serves as a precursor for the synthesis of prostaglandins and leukotrienes (2). PLA_2s are classified into three main families including the Ca^{2+}-dependent secretory PLA_2s, the cytosolic PLA_2s, and the Ca^{2+}-independent phospholipases (3). The phospholipase A_2 group IIa (PLA2g2a) isoform belongs to the family of secretory PLA_2 ($sPLA_2$). Elevated levels of PLA2g2a are observed in many diseases associated with inflammation including rheumatoid arthritis, pancreatitis, and septic shock. PLA2g2a contributes to the development of atherosclerosis (4, 5). It not only acts on membrane phospholipids but also targets lipoproteins and dietary phospholipids. Consistent with its role in inflammation, PLA2g2a is expressed in macrophages, but it is also highly expressed in hepatocytes (6–8). Expression of the PLA2g2a gene is induced by cytokines including TNFα, IL-1β, and IL-6 (9, 10). At the transcriptional level, PLA2g2a is stimulated by the nuclear factors NF-KB and CCAAT enhancer-binding protein β (C/EBPβ) (11–14). Two NF-KB binding sites and a C/EBPβ-binding site have been identified in the rat PLA2g2a promoter (12).

Hypothyroidism is associated with low grade inflammation and hyperlipidemia (15, 16). Clinical studies have correlated hypothyroidism with elevated risk of hepatic steatosis and atherosclerosis (17, 18). Thyroid hormone (T_3) modulates lipid metabolism, plasma lipids, and cardiovascular function (19). T_3 exerts its physiological actions through two T_3 receptor isoforms: TRα and TRβ (20). TRs bind primarily as heterodimers with retinoid X receptor (RXR) but can bind as homodimers to thyroid response elements (TREs) in the promoters of target genes. Consensus positive TREs contain a direct repeat of the AGGTCA-like motif separated by four nucleotides (DR4). The classical view is that the unliganded TRβ binds DNA and recruits corepressors like silencing mediator of retinoid and

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[2] The abbreviations used are: PLA_2, phospholipase A_2; $sPLA_2$, secretory PLA_2; C/EBP, CCAAT enhancer-binding protein β; TR, T_3 receptor; RXR, retinoid X receptor; TRE, thyroid response element; nTRE, negative TRE; SMRT, silencing mediator of retinoid and thyroid hormone receptor; NCoR, nuclear hormone receptor corepressor; CBP, cAMP-responsive element-binding protein-binding protein; FP, forward primer; RP, reverse primer; PTU, propylthiouracil; PEPCK, phosphoenolpyruvate carboxykinase; CPT, carnitine palmitoyltransferase; luc, luciferase; DBD, DNA-binding domain; LBD, ligand-binding domain; TSH, thyroid-stimulating hormone; PLA2g2a, phospholipase A2 group IIA.

thyroid hormone receptor (SMRT), nuclear hormone receptor corepressor (NCoR), and histone deacetylases. T_3 binding leads to recruitment of coactivators such as the steroid receptor coactivator (SRC-1) and the cAMP-responsive element-binding protein-binding protein (CBP/p300) (21). Although one activity of liganded TR is to stimulate transcription, an important regulatory role of T_3 involves gene repression (22, 23). Examples of genes inhibited by T_3 include TSHα, TSHβ, Necdin, and others (24). Interestingly, T_3 suppresses many genes in liver (25).

Unlike transcriptional stimulation, the mechanisms by which T_3 represses gene expression are not well understood. Several mechanisms have been proposed to explain the TR-mediated gene repression (26, 27). However, despite considerable effort, no consensus mechanism has been established. In contrast to positive TREs, the sequence requirements of negative TRES (nTRE) are poorly defined. The nTREs, which were identified in the promoters of genes like TSHα and TSHβ, are near the transcription start site and loosely resemble the TRE half-site (28–30). It remains to be elucidated whether it is the TRE sequence or the promoter environment that is critical for negative T_3 actions.

There have been very few reports linking T_3 with the expression of PLA_2. One group found that PLA2g2a expression in astrocytes was reduced by T_3 (31). In addition, decreased expression of PLA2g2a was detected on a gene array analyzing hypophysectomized rats treated with T_3 (32). In this study, we sought to characterize the regulation of PLA2g2a in liver by T_3 and to identify the mechanism by which T_3 inhibits PLA2g2a expression. We showed that in rats the thyroid status modulated the expression of PLA2g2a and other $sPLA_2$ isoforms. Hyperthyroid rats had significantly lower levels of PLA2g2a as compared with hypothyroid rats. T_3 decreased the basal levels of PLA2g2a and blocked the cytokine-mediated induction of PLA2g2a. Mechanistically, we have identified a negative TRE in the PLA2g2a promoter and found that the coactivator CBP enhanced the unliganded TRβ-mediated induction of PLA2g2a. We demonstrated that the corepressors SMRT and NCoR1 contribute to the T_3-mediated inhibition of PLA2g2a. Our studies provide a cellular mechanism by which T_3 inhibits PLA2g2a expression.

MATERIALS AND METHODS

Cloning of rat PLA2g2a Promoter—Genomic DNA was isolated from a rat tail using Qiagen genomic DNA isolation kit (51304). The promoter region was obtained from the PCR amplification of the genomic DNA. The forward primers contain SacI restriction sites, whereas the reverse primers contain a BglII restriction sites. The PCR products were cut with appropriate restriction enzymes and were cloned in to PGL4 expression vector.

Transient Transfections—HepG2 cells were transfected by the calcium phosphate method as described previously (33). Transfections included 2 μg of PLA2g2a luciferase reporters, 1 μg of SV40-TRβ, and 0.1 μg of TK-*Renilla*. On the next day, the cells were washed twice with PBS, and the medium was changed to serum-free medium, and T_3 was added. The cells were harvested after 24 h, and luciferase assays were conducted using the Promega dual luciferase kit (catalog no. E 1960). Luciferase values were normalized for protein content and *Renilla* luciferase activity to account for cell density and transfection efficiency, respectively.

Real Time PCR—RNA was isolated with RNA-Stat-60 (Tel-Test). Isolated RNA was further purified with the Qiagen RNeasy mini kit (74104) and quantified using a NanoDrop machine (Thermo Scientific). RNA (2.5 μg) was reverse transcribed using Superscript III (Invitrogen). The resulting cDNA was diluted 1:5 in nuclease-free water for real time PCR reactions. The parameters for real time PCR were as follows: 95 °C for 5 min and 40 cycles of 95 °C 15 s, 60 °C 30 s, and 72 °C 10 s. The final concentration of primers in each well in the PCR plates was 0.1 μM. The target genes were normalized with the 18 S gene. PCR products were quantified using the $\Delta\Delta C_t$ method. The forward (FP) and reverse primers (RP) used for real time PCR are as follows: rat PLA2g2a FP, catggcctttggctcaattcaggt; rat PLA2g2a RP, acagtcatgagtcacacagcacca; rat PLA2g3 FP, acagccttgaacttctggtccact; rat PLA2g3 RP, gctttgagcaggttgaagcgttg; rat PLA2g5 FP, aactgtgtggtcttgaacctccgt, rat PLA2g5 RP, acacactctcatgcagcctaccat; rat PLA2g1b, Qiagen QT00179529; human PLA2g2a FP, catggcctttggctcaattcaggt; and human PLA2g2a RP, aggctggaaatctgctggatgtct. Primers for the carnitine palmitoyltransferase (CPT) 1a, pyruvate dehydrogenase kinase 4, phosphoenolpyruvate carboxykinase (PEPCK), and SREBP-1c genes have been reported previously (34).

ELISA—HepG2 cells were plated in 24-well plates at a density of 0.2×10^6. The following day, serum-free DMEM was added to the cells. After 24 h, cell culture medium was collected in Eppendorf tubes and centrifuged at 1200 rpm to ensure that the cell supernatant was free of cell debris. ELISA was performed with the $sPLA_2$ (human Type IIA) EIA kit from Cayman Chemicals (585000).

Primary Rat Hepatocyte Cell Culture and Treatment—Rat hepatocytes were prepared by collagenase perfusion as described previously (35). Hepatocytes (3×10^6 in 60-mm dishes) were maintained for 12 h in RPMI 1640 medium and 10% fetal bovine serum. Following two washes with PBS, the medium was replaced by RPMI 1640 medium without serum. The cells were treated with 100 nM T_3 for 24 h. TNFα was added to the cells at a concentration of 25 ng/ml. For mRNA stability determinations, 5 μg/ml of actinomycin D was used.

Animals and Treatments—Adult male Sprague-Dawley rats were housed under controlled conditions (22 °C, constant humidity, 12-h light/12-h dark cycle) in the animal care facility of the University of Tennessee Health Science Center. Hypothyroidism was induced by feeding an iodine-free diet containing 0.1% propylthiouracil (PTU) (Teklad 95125) for 5 weeks. The rats were given intraperitoneal injection of T_3 (0.33 mg/kg of body weight) (36). After 24 h, another bolus of 0.33 mg/kg T_3 was given. Free T_3 levels in serum were measured at the University of Tennessee Endocrinology Laboratory. T_3 levels were 1.1 and 45 pg/ml in hypothyroid and hyperthyroid animals, respectively. The rats were sacrificed after 24 h, and the livers were isolated for RNA and protein.

Electrophoretic Mobility Shift Assays—To conduct electrophoretic mobility shift assays, double-stranded oligonucleotides were labeled with Klenow enzyme and [α-^{32}P]dCTP. Recombinant histidine-tagged TRβ and RXRα were prepared

in the BL21 *Escherichia coli* strain as described previously (33). Oligonucleotides contained sequences representing the nTRE. The protein-DNA binding mixtures contained labeled probe (60,000 cpm) in 80 mM KCl, 25 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and poly(dI-dC). The binding reactions were incubated at room temperature for 20 min and then resolved on 5% nondenaturing acrylamide gels in Tris-glycine buffer (22 mM Tris and 190 mM glycine) (33).

Site-directed Mutagenesis of the PLA2g2a Promoter—The QuikChange XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) was used to alter nucleotides in the nTRE in the −448/+58 PLA2g2a-luciferase vector. The sequences of the forward primers used in the mutagenesis reactions were: −102Mut, ccgtctgtgaatccatgcgcagggcacacccacctcc; −97Mut, ccgtctgtgaatccatgcgcaggccacacccacctcc; −92Mut, cgtctgtgaatccattattttatagcacccacctccccatccctg; −87Mut, gtgaatccattctttggccaagataacctccccatccctgtggc; and −82Mut, cattatttggccacaccctatgtcccatccctgtggctctc.

Knockdown Experiments—siRNA against human SMRT and NCoR1 and RNA interference-negative control were purchased from Dharmacon (Lafayette, CO). HepG2 cells were transfected with the siRNA against SMRT (L-020145-01), NCoR1 (L-003518-00), or nonspecific siRNA (D-001810-10-20) using Lipofectamine 2000 (Invitrogen). Knockdown of SMRT and NCoR1 was confirmed by real time PCR and Western blot. After 16 h of transfection, the cells were treated with 250 nM T_3 in serum-free medium for 24 h. Forty-eight hours after transfection, the cells were harvested for RNA and proteins.

Western Blot—Western blot analysis was performed on whole cell extracts from HepG2 cells and rat hepatocytes (37). The cells were harvested in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA pH 8.0, 1% Triton, 1 mM benzamidine, 0.5 mM PMSF, and protease inhibitor mixture from Sigma). The cells were kept on ice for 30 min. Cell debris was removed by centrifugation at 12,000 rpm for 20 min at 4 °C. Protein was quantified by BCA method. An equal amount of protein was loaded on a 3–8% Tris acetate acrylamide gel and transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad). The membranes were immunoblotted with primary antibodies NCoR1 (5948; Cell Signaling), SMRT (06-891; Millipore), and actin (A3853; Sigma) in Tris-buffered saline with Tween 20 containing 5% nonfat dry milk powder. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody. Immunoreactive proteins were detected using Supersignal West Femto Chemiluminescent Substrate (Thermo Scientific).

Immobilized Template Assays—PLA2g2a core promoter fragment −119/+58 and the +1108 to +1256 control region were PCR-amplified from genomic DNA using the 5′ biotinylated forward primer and the reverse primers. The template DNA was purified with a gel extraction kit (Qiagen; M-280). Streptavidin Dynal beads (Invitrogen) were resuspended in equilibration buffer (5 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 M NaCl) and then conjugated with 70 ng of biotinylated template for 30 min at room temperature with constant agitation. The immobilized templates were concentrated with a magnetic particle concentrator, washed once with 5 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 M NaCl, 0.05% Tween 20, and subsequently with binding buffer (20 mM HEPES, pH 7.6, 4 mM $MgCl_2$, 80 mM KCl, 0.08 mM EDTA, 8 mM DTT, 10% glycerol, and 0.05% Tween 20). The beads were concentrated by magnetic separator and resuspended in 25 μl of binding buffer along with TRβ, RXRα, poly(dI-dC), and 100 nM T_3 for 20 min at room temperature. The Dynal beads were washed four times with binding buffer, resuspended in 3× SDS loading buffer, resolved by 4–12% Bis-Tris gel, and analyzed via immunoblotting using TRβ antibody (sc-738).

Chromatin Immunoprecipitation Assays—ChIP assays were conducted according to manufacturer's protocol Millipore Magna ChIP kit (17–610) with minor modifications. Rat hepatocytes were grown in RPMI 1640 medium containing 5% fetal bovine serum and 5% calf serum for 24 h. The cells were treated with 100 nM T_3 overnight in serum-free medium. The next day, cells were cross-linked with 1% formaldehyde for 10 min at room temperature and sonicated as previously described (37) to give DNA fragments between 500 and 800 bp. The supernatant chromatin was precleared and immunoprecipitated with the control antibody IgG (sc-2027; Santa Cruz), anti-TRβ (MA1–216; Thermo Scientific), anti-NCoR1 (5948; Cell Signaling), or anti-SMRT (17-10057; Millipore) overnight at 4 °C along with magnetic protein G beads. The beads were washed, and the DNA was eluted. Eluted DNA was purified using the PCR purification kit (28104; Qiagen). DNA was subjected to 32 cycles of PCR using 3–5 μl of DNA. PCR products were analyzed on 2% Nusieve 3:1 agarose (Lonza, Walkersville, MD) and visualized with a Multimage light cabinet with Alpha Imager EP software. The following primers were used to amplify the 180/+61 PLA2g2a promoter: PLA2g2a FP, gcccgtctgtgaatccattatttggc; and PLA2g2a RP, caaatgcatccaaagggcaggagt. For the downstream +1639/+1900 control region, the primers were PLA2g2a FP (cacacacatgcatgctgggaactt) and PLA2g2a RP (gcttaggctgcctttgagttctct).

RESULTS

Thyroid Hormone Inhibits PLA2g2a Expression—Our first experiments characterized the regulation of PLA2g2a gene expression by T_3. We treated rat hepatocytes and human HepG2 cells with 100 nM T_3 for 24 h and measured the mRNA and protein abundance of PLA2g2a. In hepatocytes, there was a 48% decrease in the PLA2g2a mRNA levels (Fig. 1*A*). To rule out the possibility of nongenomic effects of T_3, we conducted a time course study for PLA2g2a mRNA abundance. No rapid T_3 effect was observed, and a significant reduction in PLA2g2a mRNA levels was seen after 12 h (Fig. 1*B*). These results suggested that the actions of T_3 on PLA2g2a involved genomic mechanisms. PLA2g2a protein abundance was reduced nearly 70% by T_3 (Fig. 1*C*). We tested whether T_3 could decrease the mRNA stability of PLA2g2a by adding actinomycin D. The rate of mRNA decay was identical for actinomycin D and actinomycin with T_3, suggesting that T_3 does not impact the mRNA stability (Fig. 1*D*). To examine whether T_3 had similar effects in human hepatoma cells, HepG2 cells were treated with T_3. Both the levels of mRNA as well as secreted PLA2g2a protein in cell media were decreased 50% (Fig. 1, *E* and *F*).

Thyroid Hormone Decreases the Cytokine-mediated Induction of PLA2g2a—We investigated whether T_3 could block the cytokine-mediated induction of PLA2g2a. We treated rat hepa-

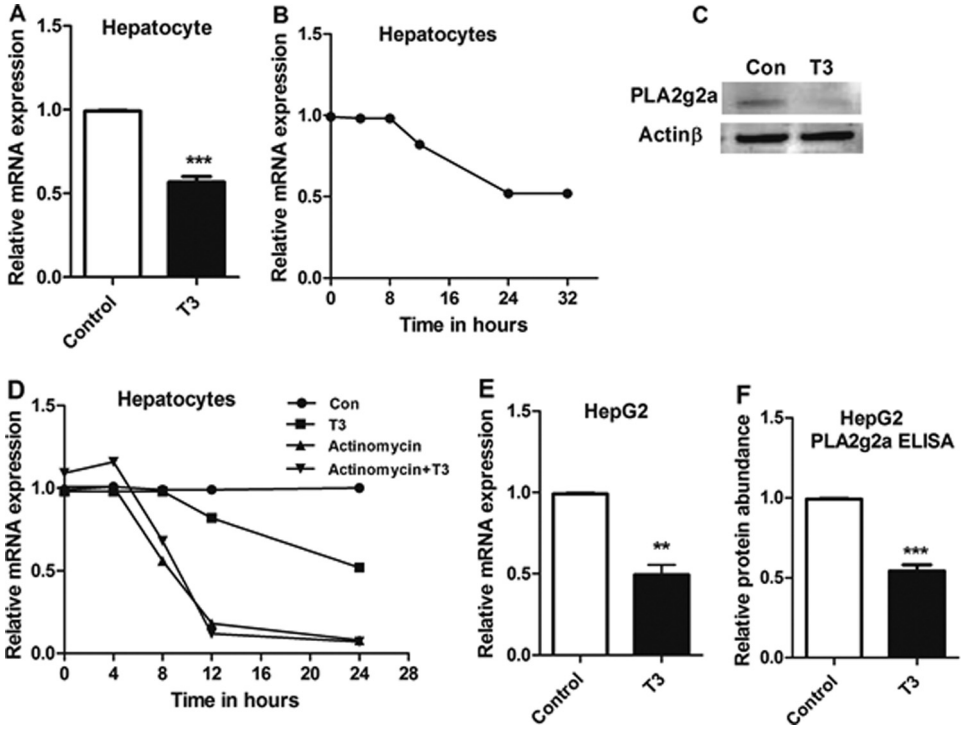

FIGURE 1. **T_3 decreases $sPLA_2$ expression.** *A*, rat hepatocytes were treated with 100 nM thyroid hormone (T_3) for 24 h. PLA2g2a mRNA levels were measured by real time PCR. *B*, rat hepatocytes were treated with T_3 for various times, and PLA2g2a RNA abundance was measured. *C*, PLA2g2a protein in rat hepatocytes was assessed by Western blotting after 24 h T_3 treatment. *D*, hepatocytes were exposed to T_3 or actinomycin D or both as in *B*. RNA was assessed at various time points. *E*, PLA2g2a mRNA levels in HepG2 cells were assessed following exposure to T_3 for 24 h. *F*, PLA2g2a protein levels in HepG2 cells were measured by ELISA. The data are expressed as the relative RNA or protein expression. All of the experiments were repeated four to six times. The data are expressed as the means of the fold induction by $T_3 \pm$ S.E. of mRNA abundance relative to untreated cells. **, $p < 0.01$; ***, $p < 0.001$. *Con*, control.

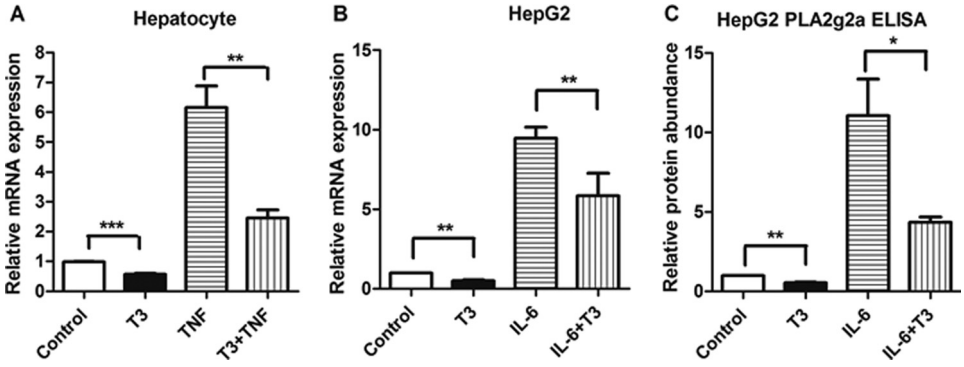

FIGURE 2. **T_3 inhibits the TNFα and IL-6 induction of PLA2g2a.** *A*, rat hepatocytes were treated with 25 ng/ml TNFα or 100 nM T_3 or both for 24 h. mRNA abundance was assessed as described for Fig. 1. *B*, HepG2 cells were treated with 10 ng/ml IL-6 or 100 nM T_3 for 24 h. RNA abundance was determined. *C*, medium was collected from HepG2 cells treated with IL-6 or T_3, and the PLA2g2a levels were determined by ELISA. All of the experiments were repeated four to six times. The data are expressed as the means of the fold induction $\pm$ S.E. of mRNA abundance relative to untreated control cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

tocytes and HepG2 cells with TNFα and IL-6, respectively. The addition of T_3 decreased TNFα induced expression of PLA2g2a mRNA from 7- to 2.5-fold in hepatocytes (Fig. 2*A*). Similarly, T_3 treatment reduced the IL-6-mediated induction of PLA2g2a mRNA from 10- to 5-fold (Fig. 2*B*) in HepG2 cells. The levels of secreted PLA2g2a protein as measured by ELISA in HepG2 cells were also decreased following T_3 treatment from 11- to 4-fold (Fig. 2*C*).

Effect of Thyroid Hormone Status on $sPLA_2$ Expression in Rats—Next, we investigated whether the thyroid status regulated endogenous $sPLA_2$ gene expression *in vivo*. The rats were made hypothyroid by providing an iodine-free diet supplemented with PTU for 5 weeks. T_3 was administered twice (0.33 mg/kg of body weight) at 24-h intervals. T_3 administration decreased the hepatic expression of PLA2g2a mRNA. Secretory PLA_2 isoforms including PLA2g1b, PLA2g3, and PLA2g5 were inhibited, suggesting that T_3 modulates additional $sPLA_2$ genes (Fig. 3, *A–D*). Expression of the lipogenic gene SREBP-1c gene was decreased by T_3 administration (Fig. 3*E*). The expression of other positively regulated genes was examined. Pyruvate dehydrogenase kinase 4, PEPCK, and CPT1a were all induced by T_3 (Fig. 3, *F–H*) (34). The abundance of the PLA2g2a protein was decreased by T_3 administration (Fig. 3*I*).

Localization of a T_3-responsive Element in the PLA2g2a Promoter—To determine whether T_3 directly regulates PLA2g2a gene expression, the rat PLA2g2a promoter (−448/+58) was

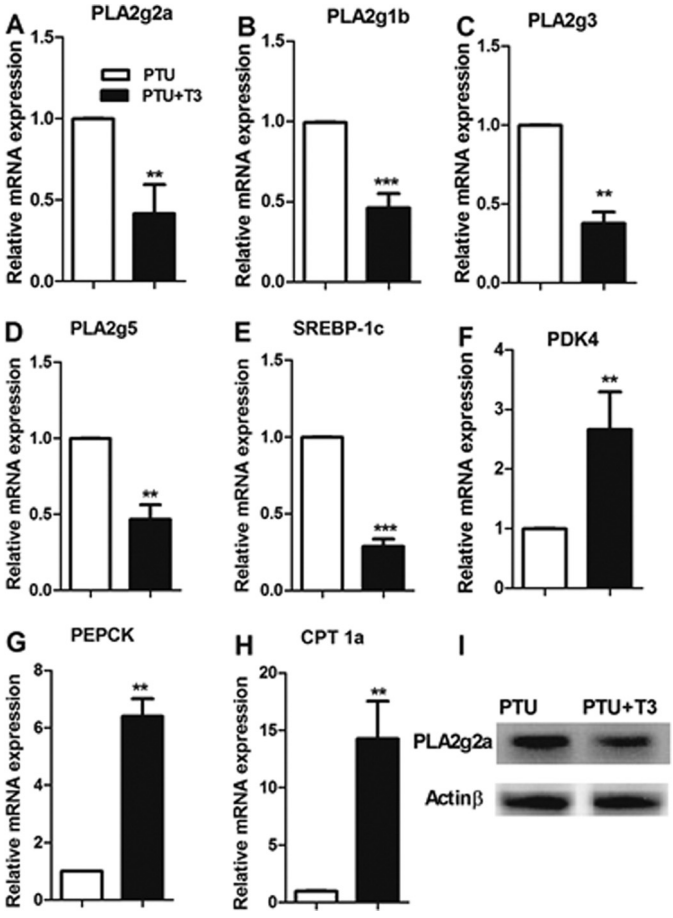

FIGURE 3. **T_3 inhibits PLA2g2a *in vivo*.** Rats were made hypothyroid with an iodine-free diet and the addition of PTU. After 5 weeks, the animals were given 0.33 mg/kg T_3, and mRNA was harvested from the liver. *A–E*, the mRNA levels were measured for PLA2g2a (*A*), PLA2g1b (*B*), PLA2g3 (*C*), PLA2g5 (*D*), and SREBP-1c (*E*). *F*, pyruvate dehydrogenase kinase 4 (*PDK4*). *G*, PEPCK. *H*, CPT1a. *I*, the protein abundance of PLA2g2a was determined by Western blotting. The values are the averages of RNA from four rats. The data are expressed as the means of the fold induction by PTU + T_3 ± S.E. of mRNA abundance relative to PTU-treated rats. **, $p < 0.01$; ***, $p < 0.001$.

cloned and fused to the luciferase (luc) reporter. The −448/+58 PLA2g2a-luc vector was transfected into HepG2 cells along with TRβ, and the cells were treated with T_3 for 24 h. T_3 decreased the activity of −448/+58 PLA2g2a-luc, suggesting that PLA2g2a is inhibited at promoter level (Fig. 4*A*). Various conflicting reports have been published on the requirement of the DNA-binding domain (DBD) for gene repression (38). To evaluate the role of the TRβ DBD, we made a TRβ mutant expressing only the ligand-binding domain (TRβ-LBD) and lacking DBD. TRβ-LBD was transfected with −448/+58 PLA2g2a-luc into HepG2 cells. The TRβ-LBD did not exhibit T_3-mediated repression (Fig. 4*A*). These data suggest that the intact DNA-binding domain is critical for the T_3 inhibition and that TRβ might bind directly to PLA2g2a promoter. To identify the T_3-responsive element, 5′ serial deletions of the PLA2g2a promoter were made. Deletion of the promoter region between −102/−82 resulted in a loss of repression by T_3, suggesting that a negative TRE was located in the −102/−82-proximal promoter region (Fig. 4*B*).

Because the DNA-binding domain of TRβ was required to repress PLA2g2a, we next tested whether TRβ binds directly to the PLA2g2a promoter using immobilized template assays. We generated a biotinylated −119/+58 fragment of the PLA2g2a promoter and a control +1108/+1256 region. The biotinylated DNA templates were incubated with the recombinant TRβ and RXRα with and without T_3. The biotinylated templates along with the bound proteins were precipitated with streptavidin beads. The beads were washed, and the bound proteins were eluted and analyzed by Western blotting using a TRβ antibody. Strong binding of TRβ was observed in the −119/+58 promoter region, suggesting that TRβ binds to the promoter. T_3 had no effect on binding. A faint signal was observed using the +1108/+1256 control region, probably because of nonspecific association of TRβ with the biotinylated control probe. (Fig. 4*C*).

Characterization of the nTRE in the PLA2g2a Gene—Next, we examined whether TRβ binds directly to the nTRE (−102/

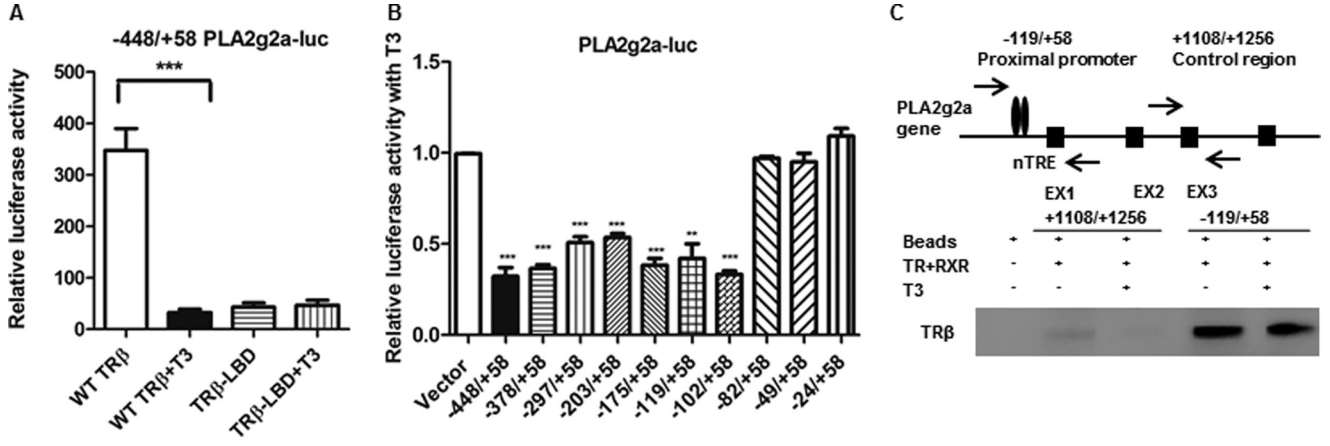

FIGURE 4. **Localization of a T_3-responsive element.** *A*, HepG2 cells were transfected with 2 μg of −448/+58 PLA2g2a luciferase, 1 μg of SV40-TRβ or TRβ-LBD, and 0.1 μg of TK-*Renilla* and treated with or without T_3 for 24 h. *B*, HepG2 cells were transfected with serial deletions of the rat PLA2g2a promoter ligated in front of the luciferase reporter gene (PLA2g2a-luc) and an expression vector for TRβ. The cells were treated with T_3 for 24 h. The data are expressed as relative inhibition with T_3. All transfections were repeated four to six times. The significance is calculated relative to the empty vector PGL4. The *error bars* indicate S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). The immobilized template assay was conducted with the biotinylated DNA corresponding to region −119/+58 and +1158/+1256 control region. *C*, schematic representation of PLA2g2a core promoter primers and control primers used to generate biotinylated DNA. The biotinylated DNA was incubated with histidine-tagged TRβ and histidine-tagged RXRα with and without T_3. The protein DNA complexes were resolved on Bis-Tris 4–12% gel and probed for His-TRβ using a TRβ antibody.

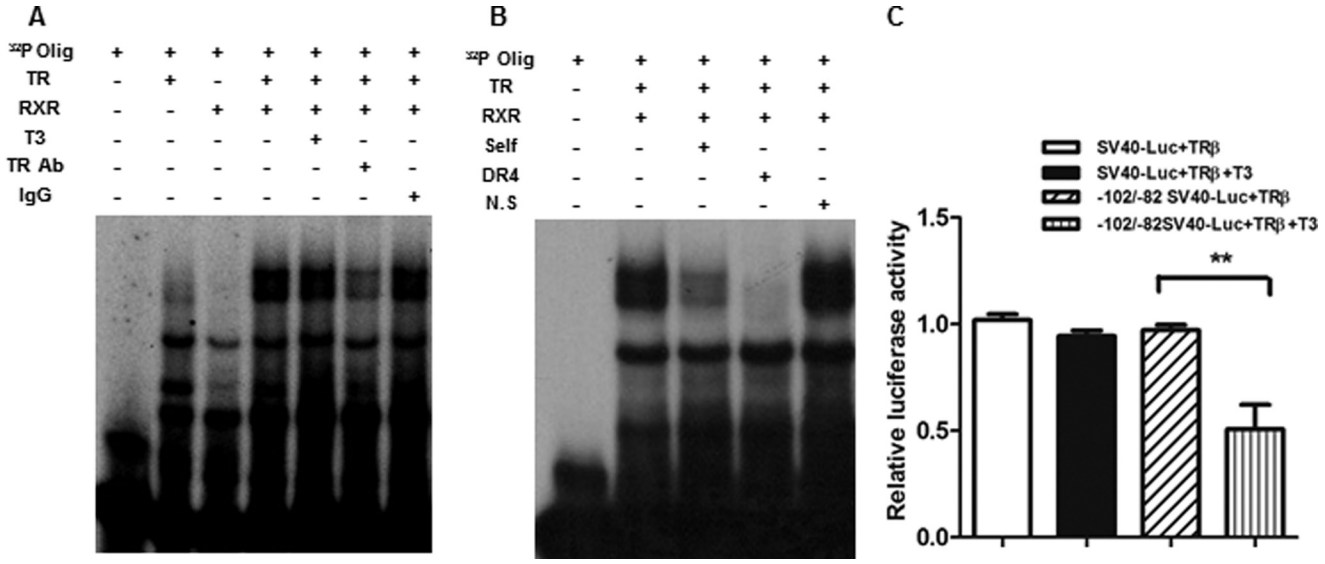

FIGURE 5. **Characterization of nTRE.** *A*, double-stranded oligonucleotides were constructed that encompassed the −102/−82 nucleotides in the PLA2g2a gene. The ^{32}P-radiolabeled double-stranded oligomer representing the −102/−82 element was incubated with purified TRβ and RXRα. Antibodies to TRβ and IgG as well as 100 nM of T_3 were added. *B*, to assess the specificity of the TR-RXRα binding, a 10-fold excess of the competitor oligomers was added. Competition assays were conducted using double-stranded unlabeled −102/−82, direct repeat of AGGTCA separated by four nucleotides (DR4) and nonspecific oligomer (*N.S*). *C*, the −102/−82 element was cloned into a luciferase reporter plasmid in front of the SV40 promoter (−102/−82 SV40-luc). This reporter was cotransfected with TRβ into HepG2 cells in the presence or absence of T_3. The cells were treated with T_3 for 24 h. The transfections were repeated four times. Luciferase activity was corrected for both protein content and *Renilla* activity. The *error bar* indicates S.E. **, $p < 0.01$.

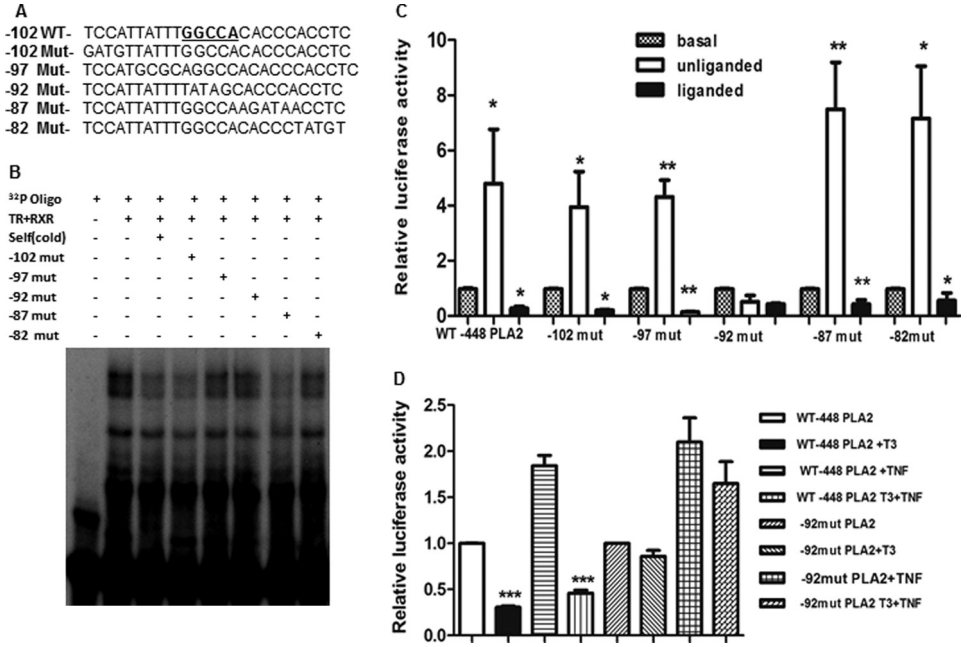

FIGURE 6. **Identification of nucleotides critical for T_3 responsiveness.** Mutations in the −102/−82 element were made to identify the nucleotides necessary for TRβ binding. *A*, the sequence of the various nucleotide substitutions is shown. *B*, competition gel shift assays were conducted with a 10-fold excess of cold −102/−82 mutants and purified TRβ and RXRα proteins. *C*, different mutants of PLA2g2a promoter corresponding to the gel shift oligomer sequences were introduced by site-directed mutagenesis. HepG2 cells were transiently transfected with 2 μg of different PLA2g2a luciferase mutants, 1 μg of SV40-TRβ, and 0.1 μg of TK-*Renilla*. The cells were treated with or without T_3 for 24 h. *D*, HepG2 cells were transfected with PLA2g2a-luc and treated with 25 ng/ml TNFα or 100 nM T_3 as described for *C*. All transfections were repeated four times. Luciferase activity was corrected for both protein content and *Renilla* activity. The *error bar* indicates S.E. *, $p < 0.05$; **, $p < 0.01$.

−82) of the PLA2g2a gene. Electrophoretic mobility shift experiments were performed using purified TRβ or RXRα proteins. Binding studies were carried out with a ^{32}P-labeled PLA2g2a nTRE oligomer. The DNA-protein complexes were resolved on a nondenaturing acrylamide gel. The results showed that neither TRβ nor RXRα alone could bind to the −102/−82 region (Fig. 5*A*, *second* and *third lanes*). TRβ along with RXRα bound as a heterodimer to −102/−82 region, whereas T_3 had no effect on binding (Fig. 5*A*, *fifth lane*). To confirm the specificity of the protein binding, antibodies to TRβ and IgG were used. The TRβ antibody disrupted the complex, showing that TRβ was present in the complex (Fig. 5*A*, *sixth lane*), whereas a nonspecific antibody had no effect (Fig. 5*A*, *seventh lane*). To check the specificity of the sequence,

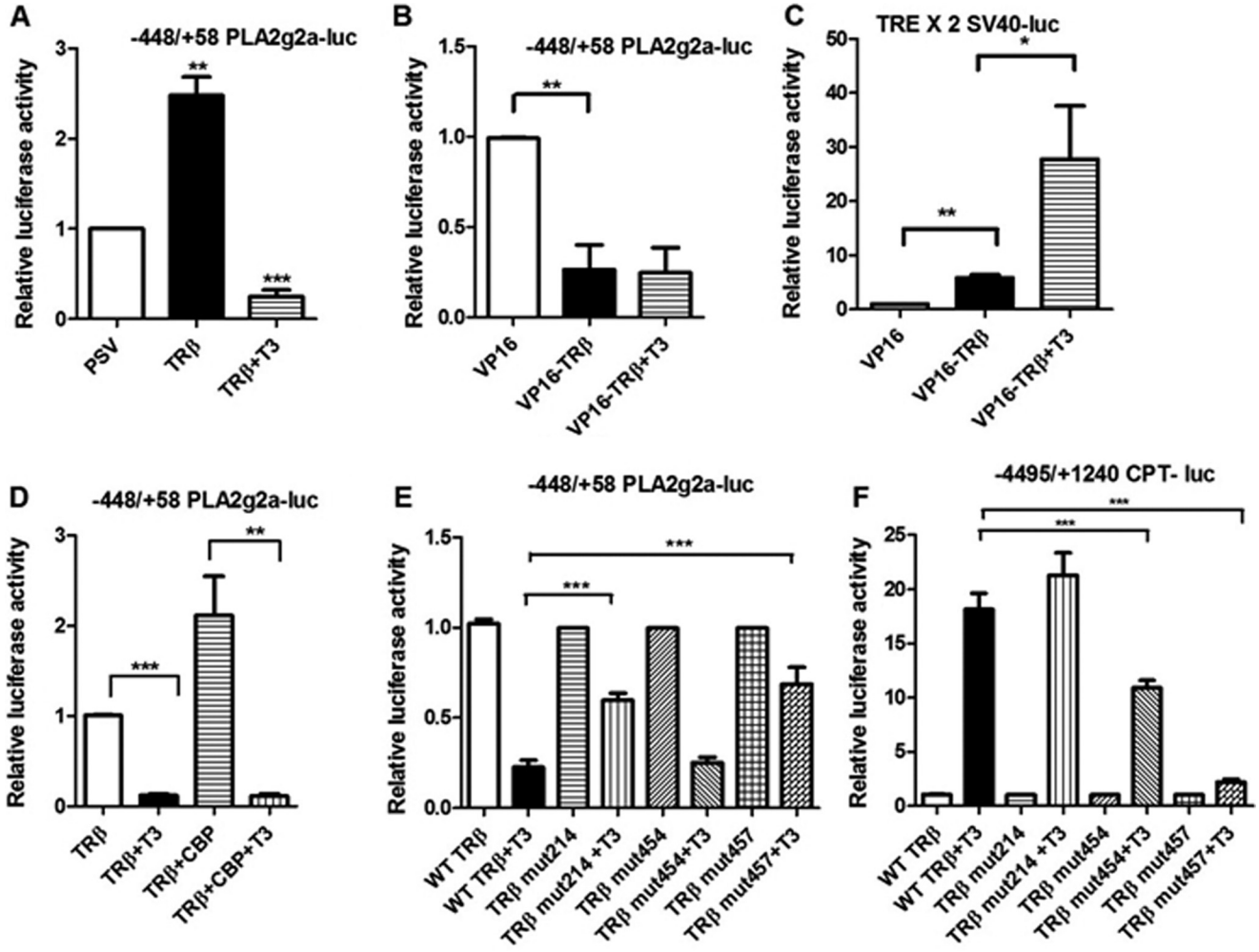

FIGURE 7. **Coactivators participate in the induction of PLA2g2a by unliganded TRβ.** *A*, HepG2 cells were transiently transfected with 2 μg of PLA2g2a luciferase reporters, 1 μg of null vector pSV-sport or SV40-TRβ, and 0.1 μg of TK-*Renilla*. T_3 was added for 24 h. *B*, HepG2 cells were transiently transfected with 2 μg of PLA2g2a luciferase reporters, 1 μg of VP16-TRβ and 0.1 μg of TK-*Renilla*. T_3 was added for 24 h. *C*, TREX2 SV40-luc was tested with VP16-TRβ as above. *D*, PLA2g2a-luc was cotransfected with expression vectors for TRβ or CBP. T_3 was added for 24 h. *E*, PLA2g2a was transfected with three different TRβ vectors carrying single amino acid substitutions. *F*, −4495/+1240 CPT1a-luc was transfected with the same TRβ expression vectors. The data are expressed as the relative luciferase activity. Luciferase activity was corrected for both protein content and *Renilla* activity. All of the experiments were repeated three to four times. The *error bar* indicates S.E. Significance is calculated relative wild type TRβ treated with T_3. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

competition assays were conducted using a 10-fold excess of double-stranded unlabeled −102/−82 oligomer, an idealized TRE (DR4), or a nonspecific unlabeled oligomer. The unlabeled −102/−82 oligomer competed with the TRβ binding (Fig. 5*B*, *third lane*), and the DR4 completely disrupted the binding, whereas nonspecific oligomers had no effect (Fig. 5*B*, *fourth* and *fifth lane*). The DR4 oligomer competed more strongly than the self −102/−82 oligomer, suggesting that the TRβ/RXRα heterodimer had more affinity for DR4 as compared with nTRE. To determine whether the nTRE conferred T_3 responsiveness to a neutral promoter, two copies of the nTRE element were ligated in front of SV40-luciferase, and this reporter was transfected into HepG2 cells. The reporter containing the nTRE was efficiently repressed in the presence of T_3 (Fig. 5*C*). These data showed that the nTRE could repress transcription in the absence of other proteins associated with the PLA2g2a promoter.

For identification of the exact nucleotides critical for TRβ/RXRα binding, we mutated the nTRE. Competition analysis was conducted using five −102/−82 oligonucleotides containing 5-bp mutations. The altered sequences are shown in Fig. 6*A*. The DNA protein complex was competed by 10-fold excess unlabeled wild type −102/−82 region (Fig. 6*B*, *third lane*). Mutants −97, −92, and −82 were unable to compete (Fig. 6*B*, *fifth*, *sixth*, and *eighth lane*), whereas mutants −102 and −87 competed (Fig. 6*B*, *fourth* and *seventh lane*). These data indicate that −97/−93, −92/−88, and −82/−79 nucleotides are involved in TRβ/RXRα binding.

Each of the mutations was introduced into the −448/+58 PLA2g2a-luc and transfected into HepG2 cells. The −92/−88 mutation eliminated the ability of unliganded TRβ to induce PLA2g2a and blocked the inhibition by T_3 (Fig. 6*C*). These data demonstrate that there is an nTRE in the promoter of the PLA2g2a gene, and the single TRE-like half-site GGCCA is critical for regulation of PLA2g2a by T_3. We tested whether the nTRE was required for the T_3 inhibition of the TNFα induction of PLA2g2a. The TNFα stimulation of PLA2g2a-luc was reduced by T_3, but expression of the PLA2g2a-luc vector with

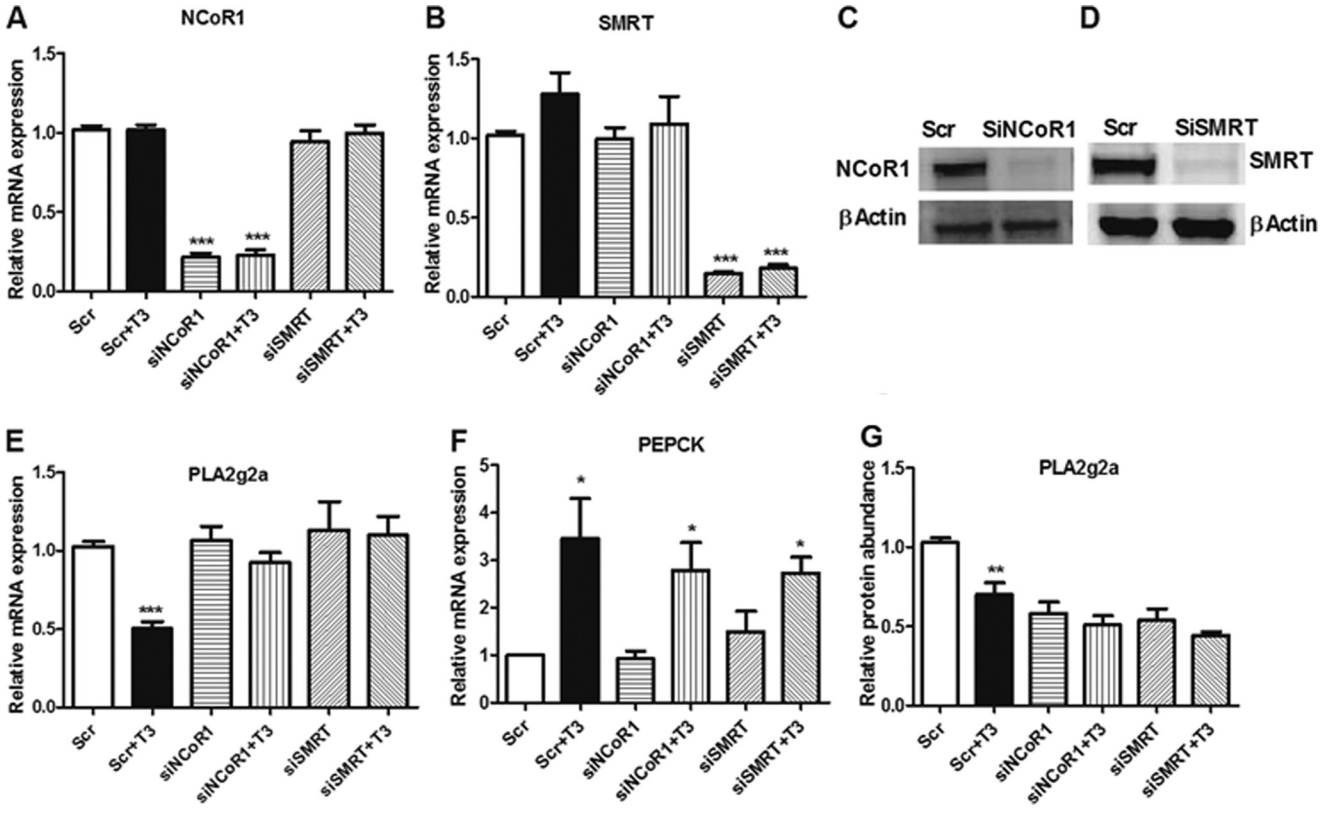

FIGURE 8. **Corepressors are involved in T_3-mediated repression of PLA2g2a.** HepG2 cells were transfected with siNCoR1 and siSMRT or scrambled siRNA overnight. The following day, the cells were treated with 100 nM T_3 for 24 h. *A*, the knockdown of NCoR1 mRNA was assessed by real time PCR. *B*, the knockdown of SMRT RNA abundance was measured. *C* and *D*, the protein abundance of NCoR1 (*C*) and SMRT (*D*) following knockdown is shown. *E*, the mRNA abundance of PLA2g2a was measured. *F*, the mRNA levels of the PEPCK gene were measured. *G*, the protein abundance of PLA2g2a in the media was measured by ELISA. The data are expressed as the relative expression ± S.E. of mRNA abundance of T_3 and untreated HepG2 cells. *, $p < 0.01$ to 0.05; **, $p < 0.01$; ***, $p < 0.001$. *Scr*, scrambled.

the disrupted nTRE (mut-92) was not blocked by T_3, indicating that T_3 inhibits cytokine action through this element (Fig. 6*D*).

Coactivators Participate in the Induction of PLA2g2a by Unliganded TRβ—Unlike positively regulated genes, some studies on negatively regulated genes suggested that unliganded TRβ is stimulatory (39, 40). To determine whether unliganded TRβ could stimulate the PLA2g2a gene, we transfected SV40-TRβ with PLA2g2a-luc. In the absence of ligand, TRβ stimulated the PLA2g2a promoter nearly 2.5-fold as compared with the basal levels (Fig. 7*A*). We next investigated the effect of a constitutively active TRβ using the full-length TRβ fused to viral protein activation domain (TRβ-VP16). PLA2g2a expression was markedly reduced by TRβ-VP16, and the addition of T_3 had similar effect (Fig. 7*B*). A DR4-containing reporter was strongly induced by TRβ-VP16 (Fig. 7*C*). These results support the concept that the activated TRβ represses PLA2g2a. We hypothesized two possible mechanisms that might be involved in PLA2g2a repression. The first possibility was a role reversal mechanism where the function of coregulators is reversed so that the coactivator causes gene repression, whereas corepressors do the opposite. Another possibility was the inverse recruitment of coregulators, *i.e.* the coactivators are associated with unliganded TRβ, whereas T_3 binding leads to corepressor recruitment. To understand the role of coactivators in PLA2g2a regulation, TRβ was transfected with coactivator CBP with and without T_3. CBP increased the TRβ-mediated induction of PLA2g2a and had no effect on T_3-dependent repression (Fig. 7*D*). This suggested the possibility that the inverse recruitment mechanism might account for T_3-mediated regulation of PLA2g2a. Furthermore, we introduced previously characterized mutations (41, 42) in the hinge region of TRβ (mut214) to reduce the interactions of TRβ with corepressors. Two other mutations in the AF-2 domain of TRβ, which were defective in coactivator binding, were made (mut454 and mut457). T_3-mediated repression of PLA2g2a-luc was relieved 50% by the TRβ mut214. In contrast this mutation had no effect on the T_3 induction of the CPT-luc. Similarly, mutations in the AF-2 domain (TRβ mut454) decreased the positive actions of T_3 on CPT-luc (Fig. 7*F*); however, it had no effect on T_3-mediated repression of −448/+58 PLA2g2a-luc. Surprisingly, the TRβ mut457 did not repress PLA2g2a (Fig. 7*E*). The corepressors and coactivators share some interactive surfaces within TRβ so that a single mutation could decrease association of both classes of coregulators (43). Overall, these data suggested that corepressors were involved in the inhibition of PLA2g2a by T_3.

T_3 Inhibition of PLA2g2a Requires Corepressors—To assess the role of corepressors in T_3-mediated inhibition of PLA2g2a, we knocked down the corepressors NCoR1 and SMRT in HepG2 cells. Knockdown of each corepressor was confirmed at the mRNA and protein level (Fig. 8, *A–D*). Knockdown of NCoR1 and SMRT reduced the ability of T_3 to inhibit PLA2g2a

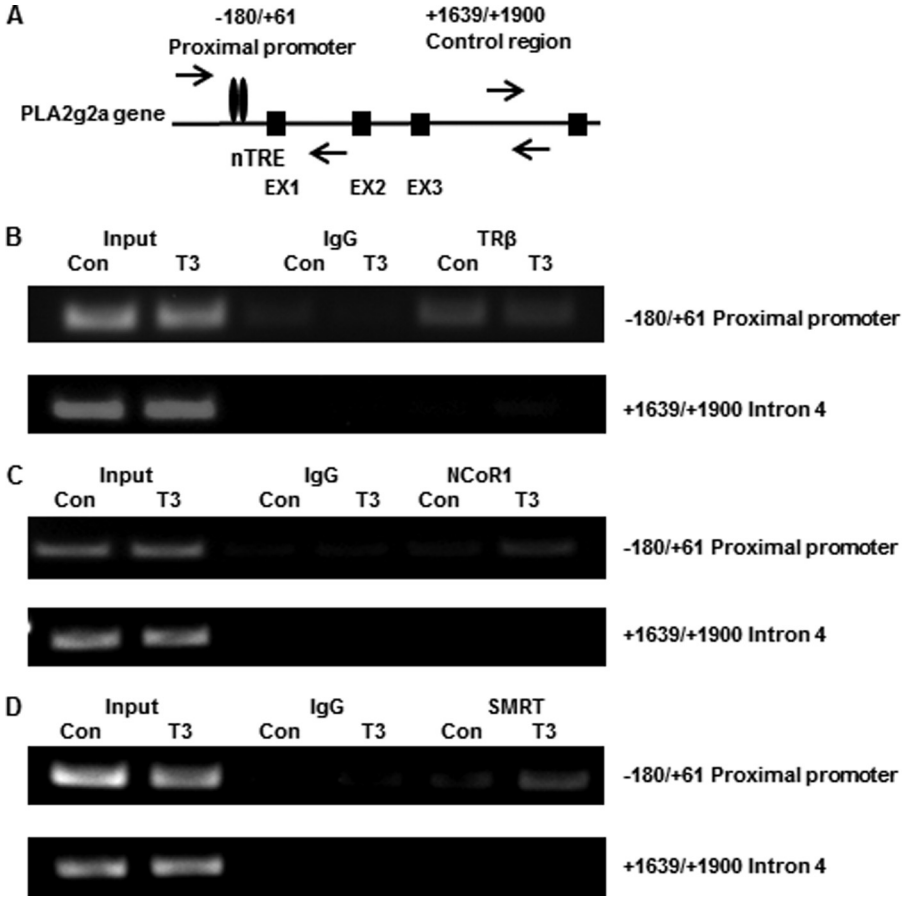

FIGURE 9. **Corepressors are recruited to the PLA2g2a gene by T_3.** Hepatocytes were treated with T_3 for 24 h. The cells were cross-linked, and the DNA was sheared for chromatin immunoprecipitation assays. *A*, a model of the PLA2g2a promoter, and the locations of the primers are shown. *B*, the TRβ antibody was used for immunoprecipitation. PCR products for the proximal promoter and the third intron are shown. *C*, ChIP experiments were conducted with an antibody to NCoR1. *D*, ChIP experiments were carried out with a SMRT antibody. *Con*, control.

mRNA and protein (Fig. 8, *E* and *G*). In contrast, T_3-mediated induction of the positively regulated PEPCK gene was not affected by corepressor knockdown (Fig. 8*F*). This experiment suggested that corepressors participate in T_3-mediated inhibition of PLA2g2a.

Corepressors Are Recruited to the PLA2g2a Gene by T_3—Because corepressors were involved in T_3-dependent repression of PLA2g2a, we next asked whether corepressors are associated with the PLA2g2a gene. We conducted ChIP assays with antibodies to TRβ, NCoR1, and SMRT. For these experiments, we used rat hepatocytes because we had identified an nTRE in the rat PLA2g2a gene. Our data indicated that TRβ is associated with the PLA2g2a promoter (Fig. 9*B*) and that addition of T_3 increased the association of corepressor NCoR1 and SMRT with the PLA2g2a gene (Fig. 9, *C* and *D*). These data support our hypothesis that T_3 suppresses PLA2g2a gene expression in part by the recruitment of corepressors.

Corepressors Are Required for the T_3 Inhibition of Other Genes—We tested whether NCoR1 or SMRT were needed for the T_3 repression of other genes by using siRNA-mediated knockdown of these corepressors in HepG2 cells. We evaluated the expression of the following 12 known T_3-responsive genes by real time PCR (24, 44): A kinase anchor protein 4 (AKAP-4), serpin peptidase inhibitor member 2 (SERPINE), solute carrier family 2 member 1 (SLC2A1), family with sequence similarity 46, member A (FAM46A), solute carrier family 26, member 3 (SLC26A3), sorbin and SH3 domain containing 1 (SORBS1), CD24 molecule (CD24), heparan sulfate 3-*O*-sulfotransferase 3A1 (HS3T3A1), KIAA1199 protein (KIAA1199), solute carrier family 1 member 4 (SLCA4), secretagogin EF-hand calcium-binding protein (SCGN), and serine/threonine kinase 17b (STK-17B). Of these T_3-repressed genes, five genes including AKAP-4, SERPINE, SLCA4, STK-17B, and HST3A1 had decreased T_3 responsiveness with corepressor knockdown, whereas others were still inhibited by T_3 (Fig 10). These data suggest that the T_3 recruitment of corepressors occurs with other negatively regulated genes. However, not all the T_3-suppressed genes utilize NCoR1 or SMRT, suggesting either redundancy of the corepressor functions or that additional mechanisms are involved.

DISCUSSION

Clinical studies have correlated hypothyroidism with elevated risk of atherosclerosis, hepatic steatosis, and components of the metabolic syndrome including hyperlipidemia and obesity (45). Chronic low grade inflammation is associated with obesity and hypothyroidism (16, 46). Secretory phospholipases enhance the progression of several chronic inflammatory diseases including arthritis and atherosclerosis (47). PLA2g2a promotes conversion of LDL to the more atherogenic oxidized

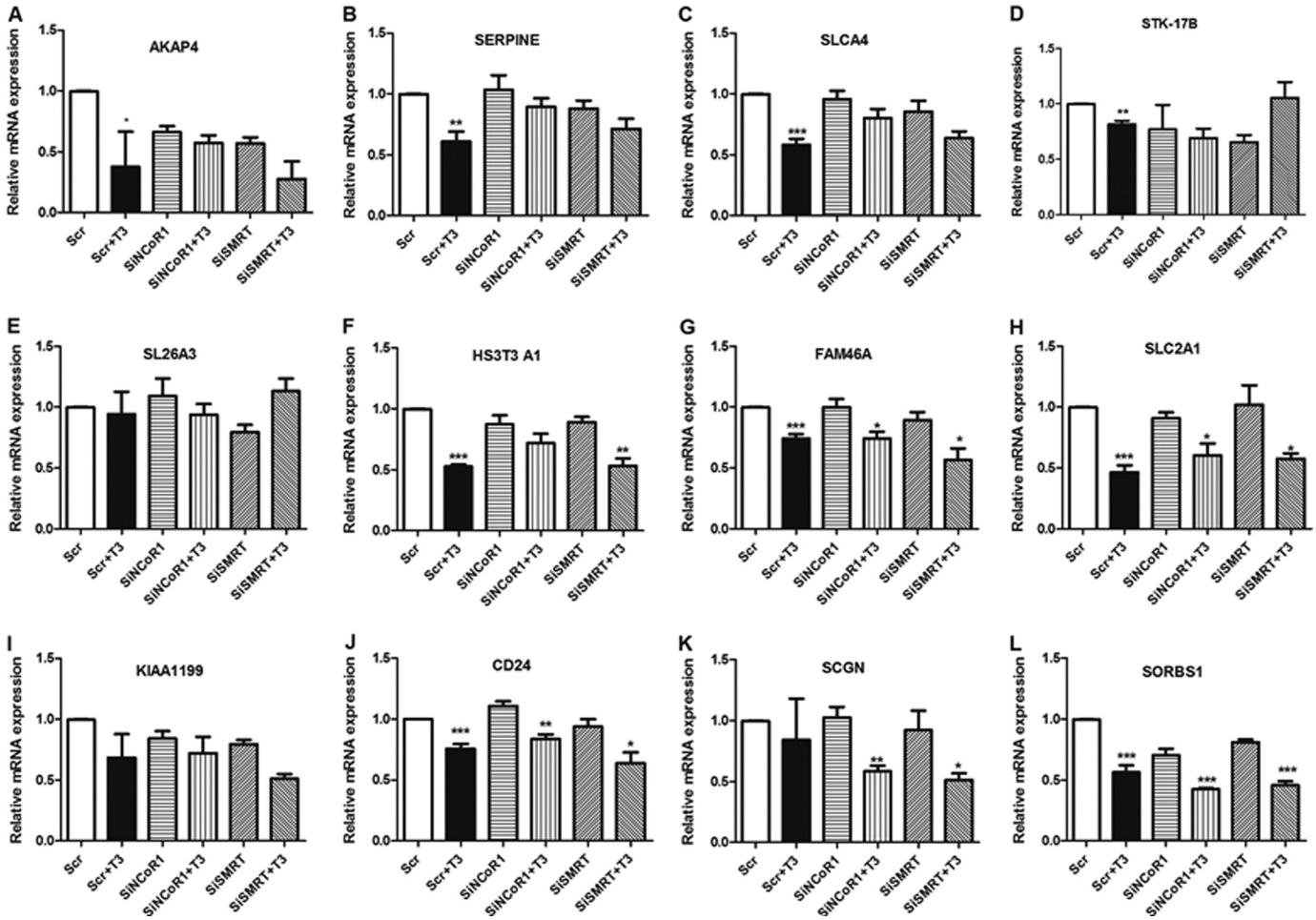

FIGURE 10. **Corepressors participate in the T_3 inhibition of several hepatic genes.** HepG2 cells were treated with siNCoR1 or siSMRT as in Fig. 8. Cells were treated with 100 nM T_3 for 24 h. The mRNA abundance was determined by real time PCR. *A*, AKAP-4; *B*, SERPINE; *C*, S SLCA4; *D*, STK17-B; *E*, SLC26A3; *F*, HS3T3A1; *G*, FAM46A; *H*, SLC2A1; *I*, KIAA1199; *J*, CD24; *K*, SCGN; *L*, SORBS1. The data are expressed as the relative expression ± S.E. of mRNA abundance of T_3 and untreated HepG2 cells. *, $p < 0.01$ to 0.05; **, $p < 0.01$; ***, $p < 0.001$. *Scr*, scrambled.

LDL (1). Most studies on PLA2g2a expression have been conducted in vascular cells and macrophages with respect to atherosclerosis and arthritis (3). However, the liver is one of the major contributors to the total pool of extracellular $sPLA_2$ (8, 48), and hepatocytes secrete PLA2g2a in response to cytokines (10, 49, 50). Because PLA2g2a expression is elevated in various inflammatory states and hepatocytes actively secrete PLA2g2a, we investigated the regulation of PLA2g2a in liver. The potential linkage of hypothyroidism with inflammation led us to examine the modulation of PLA2g2a expression by T_3. We found that T_3 inhibited the expression of PLA2g2a at the mRNA and protein level and that *in vivo* the thyroid status affected the levels of PLA2g2a. Moreover, T_3 reduced the cytokine-mediated stimulation of PLA2g2a. This inhibition of PLA2g2a is mediated through a negative TRE in the PLA2g2a promoter. TRβ binds to this nonconsensus TRE as a heterodimer with RXRα and recruits corepressors in a ligand-dependent manner.

Recent studies using PLA2g2a inhibitors have suggested that inhibition of PLA2g2a confers resistance to diet-induced obesity (51). Inhibition of PLA2g2a resulted in beneficial changes in hepatic metabolic gene expression including the induction of peroxisome proliferator-activated receptor coactivator (PGC-1α) and the inhibition of SREBP-1c. In addition, knock-out of the PLA2g1b isoform ameliorated the effects of high fat diets in part through the decreased intestinal absorption of lipids (52, 53). These studies suggest that the secretory PLA_2 isoforms contribute to the pathology of diet-induced obesity. Nonalcoholic fatty liver disease is one of the most common forms of liver disease and is characterized by accumulation of triglycerides and hepatic inflammation. Several TRβ-selective agonists including GC-1 and eprotirome have been shown to lower cholesterol and stimulate hepatic metabolism. We speculate that inhibition of PLA2g2a could be one of the beneficial therapeutic actions of selective TRβ agonists in fatty liver diseases or diet-induced obesity (45, 54, 55).

The genomic mechanisms by which T_3 induces gene expression include the ligand-mediated activation of TRβ, the dissociation of corepressors and subsequent recruitment of coactivators (21). However, many genes are repressed by T_3, and several mechanisms have been proposed for T_3-dependent inhibition of gene expression (23). The transrepression mechanism suggests that nuclear TRβ does not bind the gene promoter directly but instead interferes with the function of transcription factors. For example, T_3 inhibits hepatic ANGPTL3 gene expression via interactions of TRβ with HNF4 bound in

the ANGPTL3 proximal promoter (56). Utilizing this mechanism, the glucocorticoid receptor inhibits expression of numerous inflammatory genes via interactions with NF-KB and Fos/Jun (57). However, T_3 does not inhibit PLA2g2a through either the NF-KB or C/EBPβ binding sites, suggesting that transrepression is not the mechanism by which T_3 represses PLA2g2a. In addition, the DBD of TRβ is required for transcriptional repression.

An alternate mechanism suggests that TRβ binds to nTREs in the gene promoters repressed by T_3 (58–60). The mouse SREBP-1c gene is inhibited via a nTRE in the promoter that binds TRβ/RXRα (61). The SREBP-1c nTRE contains a single AGGTCA-like motif, but to date a consensus motif for the nTRE does not exist. Z elements, which are DNA sequences often close to transcription start sites, have been described as nTREs. A conserved Z-element sequence (CAAAG) has been delineated (62–64), but this sequence is not found within the PLA2g2a nTRE. Negative TREs do not resemble a classical positive TRE consisting of an AGGTCA motif separated by four nucleotides (DR4). The β amyloid precursor protein exhibits a variation of the nTRE in that the nTRE overlaps with Sp1 binding site. Binding of TRβ precludes binding of Sp1, thereby inhibiting Sp1-mediated induction (65). To investigate the mechanism of PLA2g2a repression, we cloned the rat PLA2g2a promoter and identified a nTRE. We provided several lines of evidence that this nTRE requires TRβ binding. First, TRβ and RXRα bind as heterodimers, as shown by gel shift assays and immobilized template assays. The TRβ mutant lacking DNA-binding domain had no effect on PLA2g2a transcription, suggesting that the DNA-binding domain is critical for the T_3 inhibition. The $-102/-82$ region was sufficient for the T_3-mediated suppression of SV40-luc. Other evidence supporting association of TRβ with this region of the promoter included ChIP assays and mutagenesis studies.

A role reversal mechanism has been proposed for a few negatively regulated genes in which a corepressor such as SMRT functions as an activator or reciprocally a coactivator represses (27). It was reported that SMRT activated the TSHα via the nTRE (66). In addition, a TR, which had a defective corepressor binding surface, was unable to activate a nTRE (67). In our studies, disruption of the corepressor-binding site of TRβ decreased the T_3 inhibition of PLA2g2a, suggesting that role reversal was not the mechanism by which T_3 acted.

For genes that are induced by T_3, the unliganded TRβ represses gene expression via the recruitment of corepressors (68). The inverse recruitment hypothesis proposes that the liganded TR recruits corepressors rather than coactivators. This hypothesis is supported by a recent study of mice harboring a mutation in the deacetylase domain of NCoR1, which was defective in interaction with histone deacetylase 3. In these mice, positive T_3-responsive genes that are normally inhibited by unliganded TR were activated, whereas negatively T_3-regulated genes like TSHα and deiodinase 2 were modestly induced by T_3 (69). This suggests that on some negatively regulated genes, liganded TR might be associated with NCoR1. In our studies, we found that the unliganded TRβ stimulated PLA2g2a transcription, leading us to speculate that coactivators were involved in the unliganded TRβ-mediated induction of PLA2g2a, whereas corepressors were associated with the liganded TRβ on PLA2g2a promoter. To validate our hypothesis, we knocked down NCoR1 and SMRT by siRNA in the T_3-treated cells. Knockdown of these corepressors alleviated the T_3 inhibition of PLA2g2a, whereas there was no effect on the T_3 stimulation of the positively regulated PEPCK gene. To determine whether corepressors are required for T_3-mediated inhibition of other genes, we investigated the effect of corepressors knockdown on other T_3-regulated genes. We found that the effect of T_3 was lost in the AKAP, STK-17B, SERPINE, and SLCA4 genes, suggesting that T_3 recruits corepressors to inhibit other genes. We believe that corepressors are recruited to the PLA2g2a gene in a ligand-dependent manner, whereas coactivators may be associated with the unliganded receptor. The basis for this inverse recruitment of corepressors by the liganded TRβ is not understood. One possible explanation is that the DNA sequence acts as an allosteric modulator for TRβ and could govern its conformation and hence determine its association with coregulators (70). It has been shown that the sequence of the binding site for the GR determines its transcriptional activity (71). In conclusion, our results demonstrate that T_3 regulates PLA2g2a gene expression *in vitro* and *in vivo* in rat liver. The nTRE in the PLA2g2a gene is sufficient for T_3-mediated inhibition. Our data indicate that T_3 represses PLA2g2a gene by an inverse recruitment mechanism in which the liganded TRβ has corepressors associated with it.

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