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Distinct modulatory roles for thyroid hormone receptors TRα and TRβ in SREBP1-activated *ABCD2* expression

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Abstract

Adrenoleukodystrophy-related protein, a peroxisomal ABC transporter encoded by *ABCD2*, displays functional redundancy with the disease-associated X-linked adrenoleukodystrophy protein, making pharmacological induction of *ABCD2* a potentially attractive therapeutic approach. Sterol regulatory element (SRE)-binding proteins (SREBPs) induce *ABCD2* through an SRE overlapping with a direct repeat (DR-4) element. Here we show that thyroid hormone (T_3) receptor (TR)α and TRβ bind this motif thereby modulating SREBP1-dependent activation of *ABCD2*. Unliganded TRβ, but not TRα, represses *ABCD2* induction independently of DNA binding. However, activation by TRα and derepression of TRβ are T_3-dependent and require intact SRE/DR-4 motifs. Electrophoretic mobility shift assays with nuclear extracts support a direct interaction of TR and SREBP1 at the SRE/DR-4. In the liver, *Abcd2* expression is high in young mice (with high T_3 and TRα levels) but downregulated in adults (with low T_3 and TRα but elevated TRβ levels). This temporal repression of *Abcd2* is blunted in TRβ-deficient mice, and the response to manipulated T_3 states is abrogated in TRα-deficient mice. These findings show that TRα and TRβ differentially modulate SREBP1-activated *ABCD2* expression at overlapping SRE/DR-4 elements, suggesting a novel mode of cross-talk between TR and SREBP in gene regulation.

Keywords: ABC transporter; Adrenoleukodystrophy-related protein (*ABCD2*); Gene expression; Peroxisome; Sterol regulatory element (SRE); Sterol regulatory protein (SREBP); Thyroid hormone; Thyroid receptor (TR); X-linked adrenoleukodystrophy (X-ALD)

Abbreviations: ABCD2/Abcd2, ATP-binding cassette transporter subfamily D member 2 gene; ALDP, adrenoleukodystrophy protein; ALDRP, adrenoleukodystrophy-related protein; DR-4, direct repeat spaced by 4 nucleotides; EMSA, electrophoretic mobility shift assay; Hprt, hydroxy-phosphoribosyl transferase; LXR, liver X receptor; PPAR, peroxisome proliferators-activated receptor; PTU, propylthiouracil; RXRα, retinoid X receptor α; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein; T_3, 3,5,3′-triiodothyronine; TR, thyroid hormone receptor; TRE, thyroid hormone response element; X-ALD, X-linked adrenoleukodystrophy.

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Introduction

X-linked adrenoleukodystrophy (X-ALD; OMIM 300100) is a severe neurodegenerative disorder that is biochemically characterized by the accumulation of very long-chain fatty acids in tissues and body fluids (Moser et al., 2001). The molecular basis for X-ALD are mutations in the *ABCD1* (*ALD*) gene, which encodes the peroxisomal ATP-binding cassette (ABC) transporter adrenoleukodystrophy protein (ALDP), with currently unknown function (Berger and Gartner, 2006; Moser et al., 2001). To date, no satisfying treatment is available for most X-ALD patients. At the molecular level, the adrenoleukodystrophy-related protein (ALDRP), encoded by the *ABCD2* gene, shows a high degree of conservation with ALDP, suggesting related or overlapping functions for ALDRP and ALDP. Indeed, upon overexpression, ALDRP can compensate for ALDP deficiency in X-ALD fibroblasts and in *Abcd1*-deficient mice (Netik et al., 1999; Pujol et al., 2004). This makes pharmacological induction of *ABCD2* an attractive therapeutic approach for X-ALD (Kemp et al., 1998), but requires detailed knowledge about regulation of the *ABCD2* gene.

ABCD2 transcription is recognized to be exceptionally complex. Next to induction by ligands of the nuclear peroxisome proliferators-activated receptor (PPAR) α and retinoid X receptor (RXR) α (Berger et al., 1999; Netik et al., 1999; Pujol et al., 2000), we have previously demonstrated that cholesterol regulates *ABCD2* expression (Weinhofer et al., 2002, 2005). This is mediated by activation of a class of transcription factors known as sterol regulatory element (SRE)-binding proteins (SREBPs), which are synthesized as membrane-bound precursors and cleaved upon induction to generate the active nuclear form. SREBPs are key regulators of cholesterol and lipid metabolism and occur as three different isoforms: SREBP1a and SREBP1c, produced from a single gene and preferentially regulating genes involved in fatty acid synthesis; and SREBP2, encoded by a separate gene and controlling expression of cholesterogenic genes (Horton et al., 2002).

Intriguingly, the SRE located in the *ABCD2* promoter overlaps with a direct repeat separated by 4 nucleotides (DR-4), which serves as binding motif for nuclear receptors and suggests cross-talk between different transcription factors (Fig. 1). Indeed, we could demonstrate that the nuclear cholesterol sensor liver X receptor (LXR) α as a heterodimer with RXRα directly binds to the DR-4 sequence and that ligand-activated LXRα interferes with SREBP1-mediated activation of the *Abcd2* promoter (Weinhofer et al., 2005). As SREBP1 and LXRα are reciprocal regulators of cholesterol metabolism but share a stimulatory effect on fatty acid synthesis, this setup could provide a mechanism for a more dynamic regulation of *ABCD2* expression.

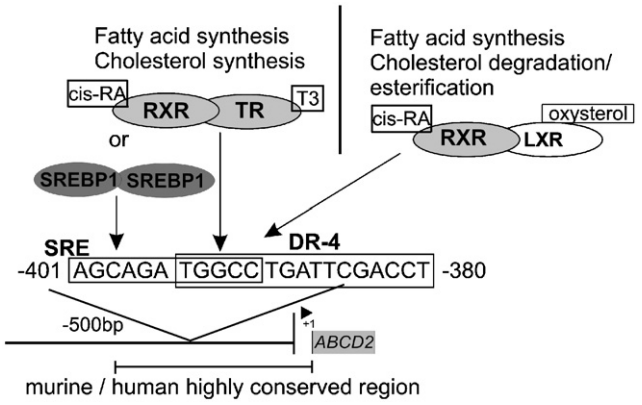

Fig. 1. The SRE and DR-4 motifs of the human *ABCD2* promoter. An SRE is located at nucleotide position −401 to −391 with respect to the translation start site of the human *ABCD2* gene. The SRE sequence overlaps by 5 bp with a direct repeat hexameric sequence of the DR-4 type (in the opposite orientation) that serves as binding site for both TRβ/RXRα and LXRα/RXRα heterodimers.

In addition to LXRα/RXRα heterodimers, also 3,5,3′-triiodothyronine (T_3) thyroid hormone receptor (TR) β homodimers or TRβ/RXRα heterodimers can bind the *ABCD2* SRE/DR-4 sequence (Fig. 1), and induction of *Abcd2* expression by T3 has been shown in cultured cells and in the liver of rodents (Fourcade et al., 2003). T_3 plays a major role in cholesterol and fatty acid metabolism by activating both TRα and TRβ isoforms, which are encoded by separate genes. Targeted mutations in mice have revealed that TRα and TRβ mediate distinct physiological effects. Whereas ablation of TRα resulted in cardiac and thermoregulatory defects, lack of TRβ led to auditory impairment and dysregulation of the pituitary–thyroid axis with increased T_3 levels (Forrest et al., 1996; Macchia et al., 2001). These distinct phenotypes seem to result from differences in expression patterns of TRα and TRβ and in the regulation of T_3 target genes (Cheng, 2005; Flamant and Samarut, 2003; Nishimura et al., 2004).

Next to activation of target genes, TRs also repress gene expression in the absence of T_3 through interaction with co-repressors that function as a platform for recruitment of subcomplexes containing histone deacetylase activity (Perissi and Rosenfeld, 2005). Moreover, also T_3-dependent inhibition of TR-regulated genes has been described, e.g. negative regulation of the hypothalamic thyrotropin-releasing hormone gene (Feng et al., 1994). The mechanism underlying this repression is poorly defined and is thought to occur by transrepression antagonizing the activities of other transcription factors (Nettles and Greene, 2005; Tagami et al., 1997).

In whole-body metabolism, SREBP1, TRα and TRβ are functionally linked through a complex transcriptional network. Many SREBP1c-regulated lipogenic genes are inducible by T_3, and a clear overrepresentation of putative SREs was observed in the promoters of T_3-regulated genes (Stahlberg et al., 2005). A direct interaction between SREBP1 and TRα while binding to adjacent SRE and DR-4 sites, respectively, is required for the T_3 response of acetyl-CoA carboxylase (*Acc*) gene expression (Yin et al., 2002). Furthermore, activation of the rat *S14* gene by T_3 and TRs was found to be dependent on the presence of an SRE in the promoter region, and a functional interaction between SREBP1 and TRs was proposed also in this context (Jump et al., 2001). Based on these results, a possible role for SREBP1 in mediating tissue-specific effects of T_3 on gene transcription was suggested (Jump et al., 2001).

This raised the question whether the T_3 response of the rat *Abcd2* gene observed by Fourcade et al. (2003) requires a functional interaction between SREBP1 and TRs. Thus, the aim of the present study was to determine the relevance of the overlapping SRE/DR-4 motif for activation of the human *ABCD2* promoter by evaluating (*i*) how the presence of either unliganded or T_3-activated TRα or TRβ affects SREBP1-stimulated *ABCD2* expression in cultured cells and (*b*) the integrated effect of SREBP1, TRα and TRβ function in vivo through analysis of *Abcd2* expression in TRα- and TRβ-deficient mice.

Materials and methods

Cell culture

The monkey kidney cell line COS-7 was purchased from ATCC. Cells were cultivated in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 50 U/ml penicillin and 100 μg/ml streptomycin (Biowhittaker).

Plasmid construction and in vitro mutagenesis

A human *ABCD2* promoter fragment was generated by PCR using a sense primer containing a KpnI site (nucleotide −2928 of GenBank™ accession code AF119822 5′-GCATCCTTCTGGGTACCATGTTC-TGCC-3′) and an antisense primer containing a SmaI site (nucleotide −56 5′-CACAGAAATCCCGGGC-AAATGTTTTAG-3′), and human genomic liver DNA as the template. The PCR fragment was cloned into the KpnI/SmaI-digested vector pGL3basic (Promega) upstream from the promoterless luciferase gene. As a positive control for T_3 response, the Moloney murine leukemia virus thyroid hormone response element (MMLV-TRE) was inserted into the expression vector pGLUC (Fourcade et al., 2003), a modified pGL2 vector (Promega) containing the minimal β-globin promoter upstream from a luciferase gene. The pairs of annealed DR-4-containing oligonucleotides with HindIII and BamHI overhangs (5′-AGCTTATCGATT-CAGGGTCATTTCAGGTCCTTGG-3′ and 5′-GATC CCAAGGACCTGAAATGACCCTGAATCGATA-3′) were ligated into HindIII/ BamHI-digested pGLUC upstream from the β-globin promoter resulting in the plasmid pGLUC-MMLV-TRE. The plasmid pGLUC-Scd1-SRE was generated by cloning annealed oligonucleotides with HindIII and BamHI overhangs (5′-AGCTTATCGATAGCAGATTGTGCATTG-3′ and 5′-GATCCAATGCACAATCTGCTATCGATA-3′) into pGLUC. To generate hABCD2 SRE/DR-4mutM1 and hABCD2 SRE/DR-4mutM2, point mutations were introduced into the DR-4 of the *ABCD2* promoter fragment by in vitro mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. PCR was conducted using the complementary degenerate primers corresponding to nucleotide −435 5′-AGCAGATGGCCTGATTCGgCCgCTCCA-AAAATAG-3′ or 5′-AGCAGATGGCCTGATTCtcttg CTCCAA-3′ introducing two or five single-point mutations, respectively. To generate a DNA-binding mutant of TRβ, point mutations were introduced into the DNA recognition domain of TRβ using the complementary degenerate primers corresponding to nucleotide 639 of GenBank™ accession code NM_000461 5′-CTACCGC-TGTATCACGTGTGGTAGTTGCAAGGGTTTCTTT-AG-3′, as described by Shibusawa et al. (2003).

Transient transfection experiments

COS-7 cells were transfected using Lipofectamine 2000 (Invitrogen) with 0.5 μg of pGL3basic or pGLUC construct, 0.1 μg human SREBP1c expression vector (Magana and Osborne, 1996), kindly provided by Dr. Timothy Osborne (University of California, Irvine, CA), 0.1 μg expression vectors encoding either the human TRα, TRβ (Fourcade et al., 2003) or TRβ DNA-binding mutant (described herein) and 0.05 μg pCMV-β-Gal (Clontech). The transfected cells were incubated for 48 h before assaying for luciferase and β-galactosidase activity using the Luciferase-β-Gal 1 step kit (Aureon Biosystems) and a Mediators PhL luminometer (Aureon Biosystems) according to the manufacturer's instructions. For ligand activation of TRs, 50 nM T_3 (Sigma) dissolved in ethanol was added 24 h post transfection.

Electrophoretic mobility shift assay (EMSA)

SREBP1a, TRα, TRβ, TRβ DNA-binding mutant, and RXRα were synthesized in vitro using the TNT T7

Quick for PCR in vitro transcription/translation system (Promega) with the primers: SREBP1a 5′-GGATCC-TAATACGACTCACTATAGGGAACAGCCACCAT-GGACGAGCCACCCTTCAG-3′ (forward), 5′-$T_{(29)}$AC TATGTCAGGCTCCGAGTCACTGCCA-3′ (reverse); TRα 5′-GGATCCTAATACGACTCACTATAGGGA-ACAGCCACCATGGAACAGAAGCCAAGCAAG-3′ (forward), 5′-$T_{(29)}$ATTAGACTTCCTGATCCTCAAA-GACCTC-3′ (reverse); TR*β*/TR*β* DNA-binding mutant 5′-GGATCCTAATACGAGTCACTATAGGGAACA-GCCACCATGACTCCCAACAGTATGACA-3′ (forward), 5′-$T_{(29)}$ACTAATCCTCGAACACTTCCAAGA AC-3′ (reverse); RXRα 5′-GGATCCTAATACGA-CTCACTATAGGGAACAGCCACCATGGACACCA-AACATTTCCTG-3′ (forward), 5′-$T_{(29)}$ACTAGGTG GTTTGATGTGGGGCCTCCAG-3′ (reverse); and the SREBP1a, TRα, TR*β*, TR*β* DNA-binding mutant and RXRα expression plasmids as templates, respectively. Nuclear extracts were prepared from COS-7 cells using the CelLytic™ NuCLEAR™ extraction kit (Sigma) according to the manufacturer's instructions. Complementary oligonucleotides corresponding to the wild-type SRE/DR-4 motif (5′-GTTCGCCAGCAGATGGCCT-GATTCGA-3′ and 5′-AGGTCGAATCAGGCCAT-CTGCTGG-3′), the SRE/DR-4mutM1 (5′-GTTCGCC AGCAGATGGCCTGATTCGG-3′ and 5′-AGCGG-CCGAATCAGGCCATCTGCTGG-3′) or the SRE/DR-4mutM2 motif from the *ABCD2* promoter (5′-GT-TCGCCAGCAGATGGCCTGATTCTCTTG-3′ and 5′-CGGCAAGAGAATCAGGCCATCTGCTGG-3′) were annealed and the double-stranded probe was labeled by filling-in the ends in the presence of [α-^{32}P]dCTP. The binding experiments were performed by pre-incubating SREBP1a, TRα, TR*β*, and RXRα synthesis mixture (or reticulocyte lysate for the negative control) with oligonucleotides as described previously (Fourcade et al., 2003). For EMSA experiments involving ligand activation of TRα or TR*β*, incubation occurred in the presence of T_3 (0.5 μM). For antibody supershift experiments, mouse anti-TRα antibody (Santa Cruz Biotechnology) was added to mixtures 30 min after the addition of the labeled probe and incubated for another 15 min at room temperature. Oligonucleotides containing both the SRE and DR-4 motif (5′-AGG-TGGTTGACCCGAGGTAACCCCTCGCATCACAC-CACCG-3′ and 5′-CCGCGGTGGTGTGATGCGAG GGGTTACCTCGGGTCAACC-3′) or only the DR-4 motif (5′-AGGTGGTTGACCCGAGGTAACCCCT-3′ and 5′-CGAGGGGTTACCTCGGGTCAAC-3′) from the chicken acetyl-CoA carboxylase (*Acc*) promoter were treated as described above and used as positive controls together with oligonucleotides corresponding to the SRE from the murine stearoyl-CoA desaturase (*Scd*) 1 promoter (5′-AGGCAGAGGGAACAGCA-GATTGTG-3′ and 5′-TCTGCACAATCTGCTGTT-CCCTC-3′).

TRα- and TR*β*-deficient mice

Two-week-old TRα-deficient (Gauthier et al., 2001) and three-month-old TR*β*-deficient mice (Gauthier et al., 1999) as well as wild-type 129/SvPas mice were used. Mice were housed, maintained and sacrificed with approval from the animal experimental committee of the Ecole Normale Supérieure de Lyon and in accordance with the "Commission de Génie Génétique" (Agreement number 12837). Briefly, control animals were maintained with mothers fed standard mouse chow and sacrificed on postnatal day 15. T_3 deficiency was induced by feeding the mothers a low iodine diet supplemented with 0.15% propylthiouracil (PTU) purchased from Harlan/Teklad. Hyperthyroidism was induced in one-half of the PTU-treated mice by daily intraperitoneal injections of a mixture of T_4 (2.5 mg per kg bodyweight) and T_3 (0.25 mg per kg bodyweight) in 100 μl PBS daily for 4 days. The animals were maintained on a 12-h day/12-h dark schedule (light on at 7 a.m.). The PTU- and thyroid hormone-treated mice were sacrificed on postnatal day 15 at 2 p.m. At the end of each experiment, the liver was quickly removed and snap frozen in liquid nitrogen. Total RNA was isolated from murine liver using TriPure isolation reagent (Roche Applied Science) and a Polytron PT3100 homogenizer (Kinematica).

Real-time quantitative PCR analysis

From total RNA, cDNA was generated by reverse transcription (GeneAmp RNA PCR Kit, obtained from Applied Biosystems) and analyzed by quantitative PCR using the iCycler iQ real-time PCR detection system (Bio-Rad). For amplification and detection of Abcd2 mRNA levels (GenBank™ accession code Z48670) the forward primer nucleotide 1959 of 5′-CACAGCG-TGCACCTCTAC-3′; reverse primer nucleotide 2032, 5′-AGGACATCTTTCCAGTCCA-3′; and the TaqMan fluorescent probe nucleotide 1986, 5′-HEX-CAAAGA-GAAGGAGGATGGGATGC-TAMRA-3′ were used. Hydroxy-phosphoribosyl transferase (Hprt) mRNA levels were used for normalization of mRNA levels. Primers for Hprt were: nucleotide 430 of GenBank™ accession code NM_013556, 5′-AAAGTTATTGGT-GGAGATGA-3′ (forward) and nucleotide 518, 5′-TGCATTGTTTTACCAGTGTC-3′ (reverse), and the fluorescent probe nucleotide 454, 5′-FAM-TCAAC TTTAACTGGAAAGAATGTCTTGA-DABCYL-3′. The standard curves for quantification were generated by serial dilution of plasmids containing murine Abcd2 cDNA (Berger et al., 1999) or Hprt cDNA (Konecki et al., 1982), kindly provided by Dr. D.W. Melton (University of Edinburgh, UK). For each assay, 12 ng cDNA was used for quantitative PCR analysis of Abcd2

or Hprt mRNA, respectively. The thermocycler was programmed: 95 °C for 10 min followed by 50 cycles at 95 °C for 20 s and 58 °C for 50 s.

Results

SREBP1-induced ABCD2 expression is attenuated by unliganded TRβ and restored by T_3-activated TRβ

Individually both, SREBP1 and TRβ homo- or TRβ/RXRα heterodimers are capable of binding the SRE/DR-4 sequence located in the *ABCD2* promoter (Fourcade et al., 2003; Weinhofer et al., 2002). Because of a 5-bp overlap between the SRE and DR-4 motifs, it is probable that steric hindrance would prevent simultaneous occupancy by SREBP1-dimers and TRβ/TRβ or TRβ/RXRα at this element (Fig. 1). To understand how presence of TRβ affects SREBP1-mediated *ABCD2* expression, co-transfection studies were performed using a luciferase reporter construct driven by a 2.8-kb human *ABCD2* promoter fragment (SRE/DR-4 wild type) and expression plasmids encoding either mature SREBP1c or TRβ. The incorporation of a plasmid encoding RXRα was not necessary because COS-7 cells endogenously express sufficient amounts of RXRα. Neither non-activated nor T_3-activated TRβ significantly influenced expression from the *ABCD2* promoter construct in the absence of SREBP1 (Fig. 2a), whereas a sevenfold induction was observed with the isolated *ABCD2* SRE/DR-4 element cloned into a minimal promoter context (data not shown) (Fourcade et al., 2003). As shown previously, SREBP1 stimulated the *ABCD2* promoter-driven luciferase expression (Weinhofer et al., 2002). However, co-transfection of SREBP1 and TRβ without ligand attenuated the SREBP1-mediated activation of the *ABCD2* promoter reporter construct, while addition of T_3 relieved this repression (Fig. 2a). In the absence of TRβ, T_3 did not affect the SREBP1-mediated stimulation of *ABCD2* expression, indicating a direct involvement of TRβ. In summary, these data suggest that unliganded TRβ suppresses SREBP1 activity on the *ABCD2* promoter.

To investigate the effect of TRβ on the binding of SREBP1 to the *ABCD2* SRE/DR-4 site more directly, we carried out EMSAs. The labeled ABCD2-SRE/DR-4 probe was shifted by co-incubation with either in vitro synthesized SREBP1 or TRβ/RXRα (Fig. 2b, lanes 2 and 3). In several independent experiments, the presence of TRβ/RXRα appeared to partially prevent binding of SREBP1 to the ABCD2-SRE/DR-4 probe (Fig. 2b, lane 4). However, it should be noted that this experimental setup does not allow quantification of binding efficiencies. Including T_3 in the binding reaction did not change complex formation (Fig. 2b, lanes 6 and 7).

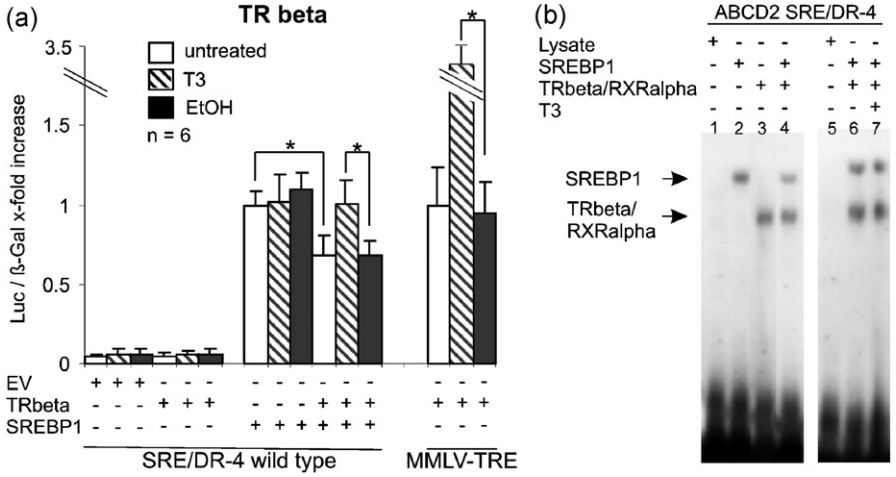

Fig. 2. SREBP1-mediated *ABCD2* expression is attenuated by unliganded TRβ and restored by addition of T_3. (a) COS-7 cells were transiently co-transfected with a luciferase reporter construct driven by a 2.8-kb human *ABCD2* promoter fragment and expression plasmids encoding the mature form of human SREBP1c and/or TRβ or the empty vector (EV) pSG5 as a negative control. Inclusion of pCMV-βGal allowed for correction of the transfection efficiency in all experiments. The DR-4 element of the Moloney murine leukemia virus (MMLV-TRE) in pGLUC served as a positive control. For ligand activation of TRβ, 24 h post-transfection cells were treated with 50 nM T_3. Statistically significant differences by Student's *t*-test are indicated by asterisks ($p<0.05$). (b) EMSA using a ^{32}P-labeled oligonucleotide probe corresponding to the human *ABCD2* SRE/DR-4 motif and in vitro synthesized SREBP1 and/or TRβ/RXRα (as indicated). Inclusion of 500 nM T_3 in the binding reaction did not change complex formation (lane 7). Incubation of the labeled probe with unprimed reticulocyte lysate did not result in a DNA–protein interaction (lanes 1 and 5). Specific bands are marked by an arrow. Representative autoradiographs of at least two independent EMSA experiments are shown.

Unliganded TRβ interferes with SREBP1 stimulation of the ABCD2 promoter through both SRE/DR-4-dependent and -independent mechanisms

It has previously been suggested that a direct interaction of TRs with DR-4 elements might not be required for negative regulation of a subset of TR target genes (Tagami et al., 1997, 1999). Thus, we investigated whether in the case of *ABCD2*, the DR-4 element is required for unliganded TRβ to attenuate SREBP1 activity. We generated two different mutated versions of the ABCD2 SRE/DR-4 sequence with two (hABCD2 SRE/DR-4mutM1) or five (hABCD2 SRE/DR-4mutM2) point mutations, respectively, in the DR-4 half site that does not overlap the SRE sequence (Fig. 3a). We tested by EMSA whether these mutations impair binding of TRβ/RXRα to the SRE/DR-4 element. As expected, incubation of a ^{32}P-labeled wild-type ABCD2-SRE/DR-4 probe with either in vitro synthesized TRβ/RXRα or SREBP1 resulted in formation of the respective protein–DNA complex (Fig. 3a, lanes 4 and 7). However, when the DR-4mutM1 version was used as a probe, complex formation was diminished and in some experiments even lost in the presence

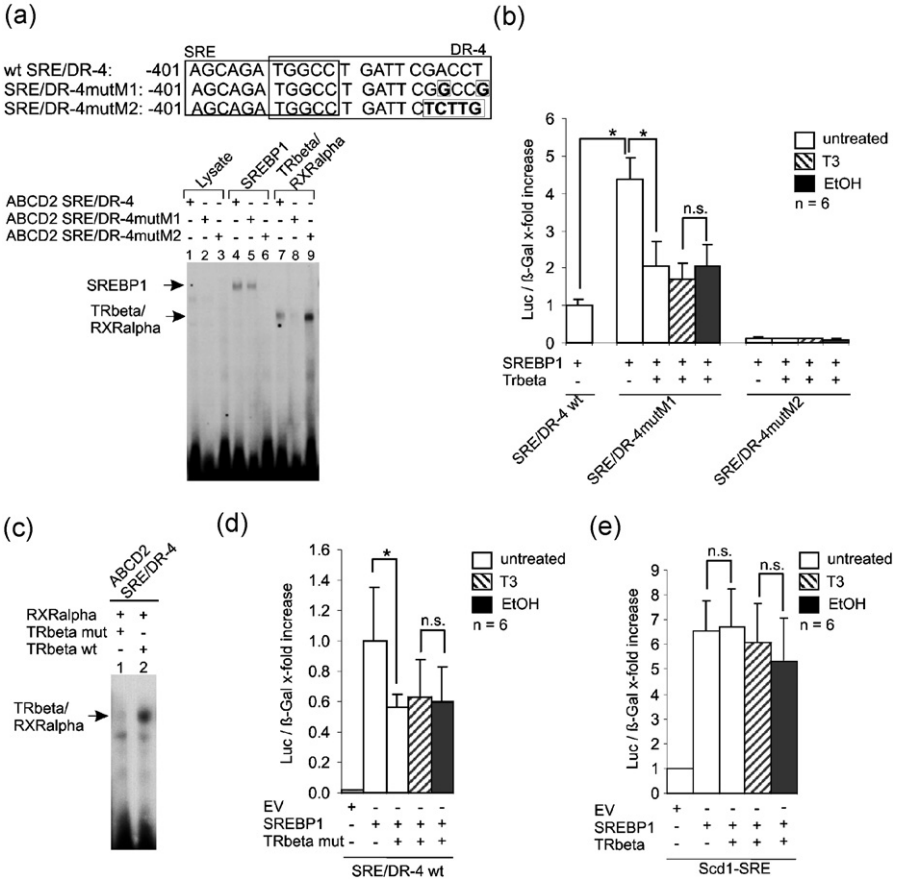

Fig. 3. Unliganded TRβ attenuates SREBP1-mediated *ABCD2* expression independent of DNA binding whereas derepression requires a DR-4 interaction. (a) EMSA using a ^{32}P-labeled oligonucleotide probe corresponding to the wild-type *ABCD2* SRE/DR-4 and either in vitro synthesized SREBP1 (lane 4) or TRβ/RXRα (lane 7) resulted in a shifted band. Using probes with two (ABCD2 SRE/DR-4mutM1) or five (ABCD2 SRE/DR-4mutM2) point mutations in the DR-4 half site outside of the SRE abolished either binding of TRβ/RXRα (lane 8) or SREBP1 (lane 6). Incubation of the labeled probe with unprimed reticulocyte lysate did not result in a DNA–protein interaction (lanes 1–3). Specific bands are marked by an arrow. (b) The DR-4 mutations M1 and M2 introduced into the *ABCD2* promoter luciferase reporter construct and co-transfected into COS-7 cells together with expression plasmids encoding SREBP1c and/or TRβ. (c) EMSA demonstrating that an in vitro synthesized TRβ mutant impaired in DNA binding is unable to bind the human *ABCD2* SRE/DR-4 motif. (d) Co-transfection of COS-7 cells using the wild-type *ABCD2* promoter-driven reporter construct and expression plasmids encoding either SREBP1 and/or the TRβ mutant impaired in DNA binding. (e) SREBP-dependent activation of the SRE from the stearoyl-CoA desaturase 1 (*Scd1*) gene in the minimal promoter context of pGLUC was not affected by TRβ in COS-7 cells co-transfected with expression plasmids encoding SREBP1c and/or TRβ. In all reporter assays, ligand activation of TRβ was performed 24 h post-transfection using 50 nM T_3. Statistically significant differences by Student's *t*-test are indicated by asterisks ($p < 0.05$). Representative autoradiographs of at least two independent EMSA experiments are shown.

of TRβ/RXRα (Fig. 3a, lane 8), indicating that the introduced mutations impair binding of TRβ/RXRα to the DR-4 sequence. For SREBP1, normal complex formation was observed using the ABCD2-SRE/DR-4mutM1 oligonucleotides (Fig. 3a, lane 5). Unexpectedly, the five different point mutations in the DR-4 sequence of the ABCD2-SRE/DR-4mutM2 probe did not prevent binding of TRβ/RXRα (Fig. 3a, lane 9) but instead completely abolished binding of SREBP1 to the SRE/DR-4 (Fig. 3a, lane 6). These results stress the importance of the 3′ flanking region, comprising a DR-4 half site, for the binding of SREBP1 in the *ABCD2* promoter context.

Thus, the two mutated versions of the DR-4 element constitute a good tool to test whether binding of TRβ to the ABCD2 SRE/DR-4 region is required for the negative effect of unliganded TRβ on SREBP1 activity. Using site-directed mutagenesis, the corresponding DR-4 mutations, SRE/DR-4mutM1 and SRE/DR-4mutM2, were incorporated into the ABCD2 promoter context. These luciferase constructs were used to transiently co-transfect COS-7 cells together with SREBP1 and/or TRβ expression plasmids in the absence or presence of ligand (Fig. 3b). In cells transfected with the SRE/DR-4mutM1 construct that binds SREBP1 but not TRβ/RXRα (Fig. 3a), a fivefold higher induction of SREBP1-mediated reporter gene expression was observed compared with the wild-type DR-4 construct (Fig. 3b). In contrast, SREBP1 was unable to activate the SRE/DR-4mutM2 construct (Fig. 3b), thus confirming functionally our finding that this DR-4 mutation abolishes the binding of SREBP1 to the SRE/DR-4 element (Fig. 3a). Next, the effect of simultaneous presence of SREBP1 and TRβ on induction of the *ABCD2* promoter was tested. Surprisingly, co-transfection with both TRβ and SREBP1 resulted in a significant reduction of SREBP1-mediated expression from the SRE/DR-4mutM1 reporter construct, indicating that the repressive effect of TRβ does not require its binding to the SRE/DR4 element. However, in contrast to the situation observed with the wild-type *ABCD2* promoter construct, addition of T_3 did not relieve the repression (Fig. 3b), implying that binding of activated TRβ to the SRE/DR-4 sequence is required for this effect.

To investigate this complicated mechanism in more detail and to exclude that TRβ exerts its repressive effect via another unidentified TRE, we generated a well-defined TRβ mutant, which is unable to bind DNA (Shibusawa et al., 2003) because two amino acids (position 125/126 of the DNA recognition motif) were exchanged by site-directed mutagenesis. As expected, the mutated TRβ was unable to bind the ABCD2 SRE/DR-4 sequence in EMSA experiments (Fig. 3c). After co-transfection of expression plasmids for the mutated TRβ and SREBP1, activation of the wild-type ABCD2 SRE/DR4 promoter reporter construct by SREBP1 was significantly impaired and could not be restored by addition of T_3 (Fig. 3d). Thus, these results indicate that unliganded TRβ downregulates SREBP1-dependent activation of the *ABCD2* promoter independently of DNA binding, while T_3-dependent reactivation of SREBP1 stimulation seems to require a direct interaction of TRβ with the SRE/DR-4 element.

The negative effect of unliganded TRβ on SREBP1-mediated transcriptional activation could possibly be exerted by a direct antagonistic protein-to-protein interaction of the two transcription factors, which might also affect other SREBP1-regulated genes. To test this hypothesis, the SRE from the murine stearoyl-CoA desaturase (*Scd1*) gene was cloned into a minimal promoter context of pGLUC and co-transfected with SREBP1 and TRβ. However, neither T_3-activated nor unliganded TRβ influenced SREBP1-stimulation of the Scd1 reporter gene construct (Fig. 3e).

Enhanced activation of the ABCD2 promoter by T_3-liganded TRα and SREBP1

We next investigated how the TRα isoform modulates SREBP1-mediated *ABCD2* expression. Thus, COS-7 cells were co-transfected with the *ABCD2* promoter reporter construct containing the wild-type SRE/DR-4 sequence and expression plasmids encoding mature SREBP1 and/or TRα (Fig. 4a). As observed for TRβ, neither unliganded nor T_3-activated TRα significantly influenced expression from the *ABCD2* promoter construct in the absence of SREBP1 (Fig. 4a), whereas a sixfold induction was observed with T_3-liganded TRα and the isolated ABCD2 SRE/DR-4 element cloned into a minimal promoter context (data not shown). When TRα was co-expressed with SREBP1 in the absence of the ligand, SREBP1 activity on the *ABCD2* promoter was not affected but, intriguingly, after addition of T_3, expression from the *ABCD2* promoter exceeded that obtained with SREBP1 alone (Fig. 4a). To verify that this positive effect on the *ABCD2* promoter requires binding of T_3-activated TRα to the SRE/DR-4 sequence, reporter gene assays were performed with the mutated DR-4 element (SRE/DR-4mutM1) that impaired binding of TRβ. In this case, T_3-activated TRα did not amplify SREBP1-stimulated luciferase activity, indicating that a direct binding of TRα to the SRE/DR-4 is required for further stimulation (data not shown). Altogether, these data show that upon ligand binding, TRα is able to enhance SREBP1-mediated stimulation of the *ABCD2* promoter in a DR-4-dependent manner.

To investigate whether a direct interaction between SREBP1 and TRα occurs at the ABCD2 SRE/DR-4 element, EMSAs were performed. We first verified that also TRα/RXRα is able to bind the human ABCD2 SRE/DR-4 sequence (Fig. 4b, lane 2). When the labeled

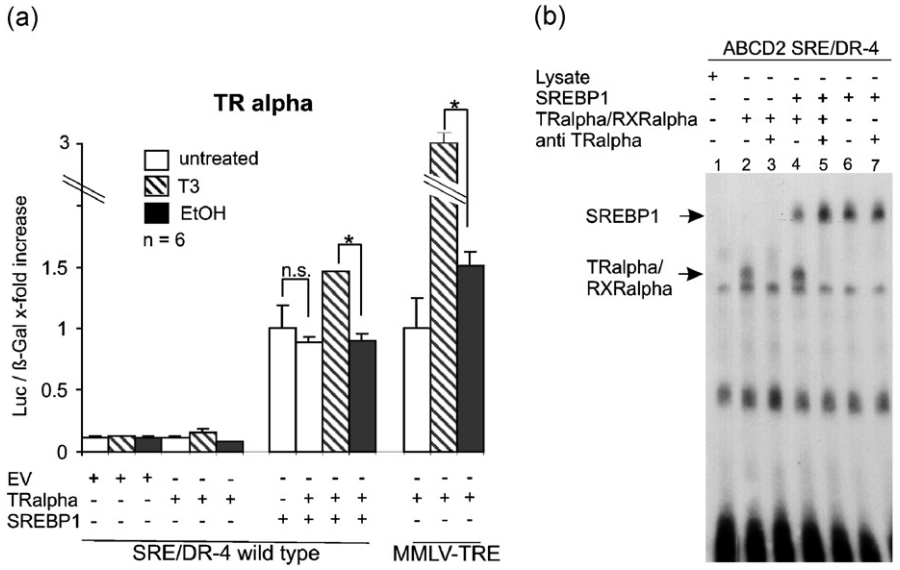

Fig. 4. Enhanced activation of the *ABCD2* promoter by T_3-liganded TRα and SREBP1. (a) COS-7 cells were transiently cotransfected with the *ABCD2* promoter reporter construct and expression plasmids encoding SREBP1c and/or TRα or the empty vector pSG5 as a negative control. The DR-4 element of the Moloney murine leukemia virus served as a positive control. Ligand activation of TRα was performed 24 h post-transfection using 50 nM T_3. Statistically significant differences by Student's *t*-test are indicated by asterisks ($p < 0.05$). (b) EMSA using a ^{32}P-labeled oligonucleotide probe corresponding to the human *ABCD2* SRE/DR-4 motif and in vitro synthesized SREBP1 (lanes 4–6) and/or TRα/RXRα (lanes 2–5). For supershift experiments, binding occurred in the presence of an anti-TRα antibody (lanes 3, 5 and 7). Incubation of the labeled probe with unprimed reticulocyte lysate did not result in a DNA–protein interaction (lane 1). Specific bands are marked by an arrow. Representative autoradiographs of at least two independent EMSA experiments are shown.

probe was concurrently incubated with SREBP1, TRα and RXRα, no additional shifted band appeared that would indicate the formation of an oligomeric complex (Fig. 4b, lane 4). Similar results were obtained in the presence of T_3, and also the incubation of the labeled probe with SREBP1 and TRα in the absence of RXRα did not result in SREBP1/TRα complex formation (data not shown). To further elucidate the impact of TRα/RXRα on SREBP1 binding, an anti-TRα antibody was included in EMSA experiments. The addition of anti-TRα antibody abolished complex formation between TRα/RXRα and the labeled SRE/DR-4-containing oligonucleotides, suggesting interference with TRα/RXRα binding to the DNA element (Fig. 4b, lanes 2 and 3). It should be noted that in some cases antibody binding causes band disappearance rather than band shift (Lee and Pedersen, 2003; Li et al., 2001). When the anti-TRα antibody was added during incubation of the labeled probe with SREBP1 and TRα/RXRα binding of TRα/RXRα was abolished, whereas binding of SREBP1 to the ABCD2 SRE/DR-4 sequence was not affected (Fig. 4b, lane 5). In summary, the results observed using in vitro synthesized proteins do not support the concept of an oligomeric complex between SREBP1 and TRα at the ABCD2 SRE/DR-4 element. However, this result might be explained by the lack of additional factors necessary for formation of such a complex.

DNA–protein interactions on the ABCD2 SRE/DR-4 in EMSAs using nuclear extracts

One of the inherent limitations of using in vitro synthesized proteins in gel shift experiments is the absence of additional cofactors that could be required to stabilize complex formation between SREBP1 and TRα on the SRE/DR-4 motif. Thus, further EMSA experiments were performed using nuclear extracts from COS-7 cells. In this experimental setup, attribution of individual complexes to the binding of a specific transcription factor is difficult, and interpretation is mainly based on comparison to the position of migrated bands generated by known DNA–protein interactions. Thus, next to the ABCD2-SRE/DR-4 probe (Fig. 5a, lane 3) additional oligonucleotides were used as standards that contained either a defined SRE (Scd1-SRE, Fig. 5a, lane 2) or a DR-4 (Acc-DR-4, Fig. 5a, lane 5) or both motifs within the same oligonucleotide (Acc-DR-4, SRE; Fig. 5a, lane 4). In the latter, which corresponds to a fragment of the chicken acetyl-CoA carboxylase (*Acc*) promoter, the DR-4 is located 4 nucleotides upstream of the SRE sequence and leads to the formation of a tetrameric structure consisting of SREBP1-SREBP1-TRα-RXRα (Yin et al., 2002).

Incubation of the COS-7 nuclear extract with the ABCD2 SRE/DR-4 probe resulted in the formation of

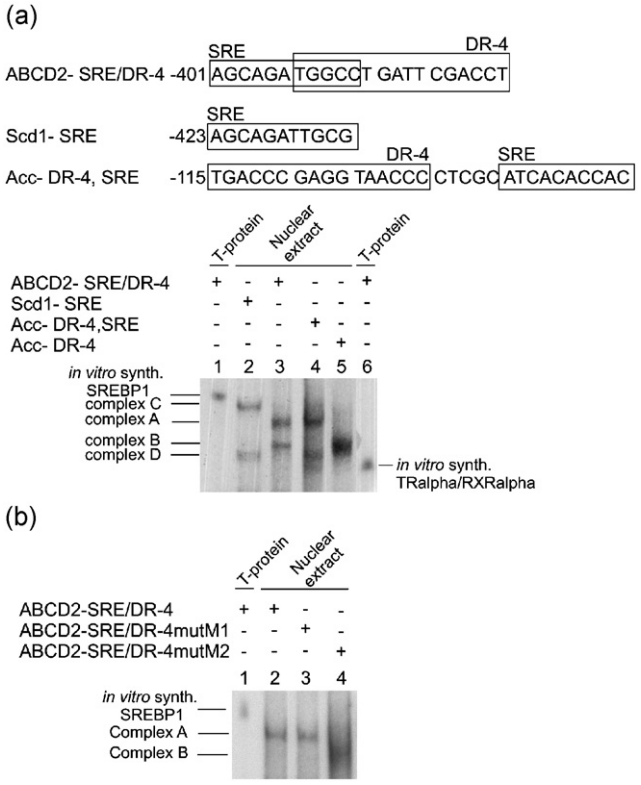

Fig. 5. DNA–protein interactions on the ABCD2 SRE/DR-4 in EMSA using nuclear extracts. (a) EMSA using ^{32}P-labeled oligonucleotide probes corresponding to the human *ABCD2* SRE/DR-4 motif (ABCD2-SRE/DR-4), the SRE from the *Scd1* promoter (Scd1-SRE), the adjacent DR-4 and SRE sequences of the *Acc* gene (Acc-DR-4,SRE) or the latter lacking the SRE (Acc-DR-4) and either in vitro transcribed/translated (T-protein) SREBP1 or TRα/RXRα or nuclear extracts derived from COS-7 cells. (b) EMSA using COS-7 nuclear extracts and a probe containing the DR-4 mutation ABCD2 SRE/DR-4mutM2 that is unable to bind SREBP1. Formation of complex A was abolished while formation of complex B was significantly strengthened (lane 4). Representative autoradiographs of at least two independent EMSA experiments are shown.

two major complexes, designated complex A and B (Fig. 5a, lane 3). Intriguingly, the ratio of these two bands varied, in that complex A always gave a strong signal whereas complex B appeared at intensities comparable to or lower than complex A. Using the Scd1-SRE-containing oligonucleotides as a probe, two DNA–protein interactions of different mobility were observed (complex C and D; Fig. 5a, lane 2). Complex C migrated slightly faster than the complex generated by in vitro synthesized SREBP1 (Fig. 5a, lane 1), probably due to binding of additional cofactors or by post-translational modifications of SREBP1. DNA–protein interactions resulting in four bands were observed using the oligonucleotides containing both the SRE and DR-4 motifs from the *Acc* promoter (Acc DR-4, SRE) (Fig. 5a, lane 4). Two of these complexes show a strong signal and mobility identical to complex A (ABCD2 SRE/DR-4 probe) and D (Scd1-SRE probe), respectively (Fig. 5a, lane 4). The two weaker bands indicate protein–DNA interactions comparable to complex C from the Scd1-SRE probe and to the complex observed with in vitro synthesized TRα/RXRα (Fig. 5a, lane 6). In the absence of the SRE sequence (Acc-DR-4), only one complex was detectable, the mobility of which was comparable to that of complex B formed with the ABCD2 SRE/DR-4 probe (Fig. 5a, lane 5). In summary, two DNA–protein interactions are formed with the ABCD2 SRE/DR-4 element and nuclear proteins: complex A, which is favored and displays a mobility solely resembling that of the complex with the Acc element containing both SRE and DR-4 but differing from those formed on DNA probes containing either SRE (Scd1-SRE) or DR-4 (Acc-DR-4); and the weaker complex B, which appears similar to the DNA–protein interaction found with the isolated DR-4 (Acc-DR-4). Thus, the results presented above are supportive of an oligomeric complex between SREBP and TRs on the ABCD2 SRE/DR-4 sequence.

In order to further prove whether SREBP1 or a DR-4-binding protein is involved in formation of complex A or B, we next used the two mutated versions of the ABCD2 SRE/DR-4 sequence, ABCD2 SRE/DR-4mutM1 and SRE/DR-4mutM2, as probes in EMSA experiments. Whereas the DR-4mutM1 sequence destabilizes binding of TR/RXR but allows binding of SREBP1, the DR-4mutM2 version retains interaction with TR/RXR but completely abolishes binding of SREBP1 (Fig. 3a). With either wild-type ABCD2 SRE/DR-4 or ABCD2 SRE/DR-4mutM1 sequences, complex A was easily detectable, whereas complex B was barely observed (Fig. 5b, lanes 2 and 3). When the ABCD2 SRE/DR-4mutM2 probe was used, complex A was no longer visible but instead formation of complex B was significantly enhanced (Fig. 5b, lane 4). Collectively, these results suggest that complex A, which formed on the ABCD2 SRE/DR-4 probe, seems to contain SREBP1 next to additional factors that are able to bind the SRE/DR-4 motif.

Lack of TRβ relieves repression of hepatic Abcd2 expression during development

Our in vitro studies disclose a complex regulation of *ABCD2* expression through modulation of SREBP1 activity by TRα and TRβ. In vivo, the two TR isoforms are differentially expressed during development. Whereas TRα mediates virtually all T_3 activity in the immature liver, TRβ is the dominating isoform in adulthood (Rodd et al., 1992). In addition, circulating T_3 levels reach a maximum early in development and decline with aging of mice (Hadj-Sahraoui et al., 2000).

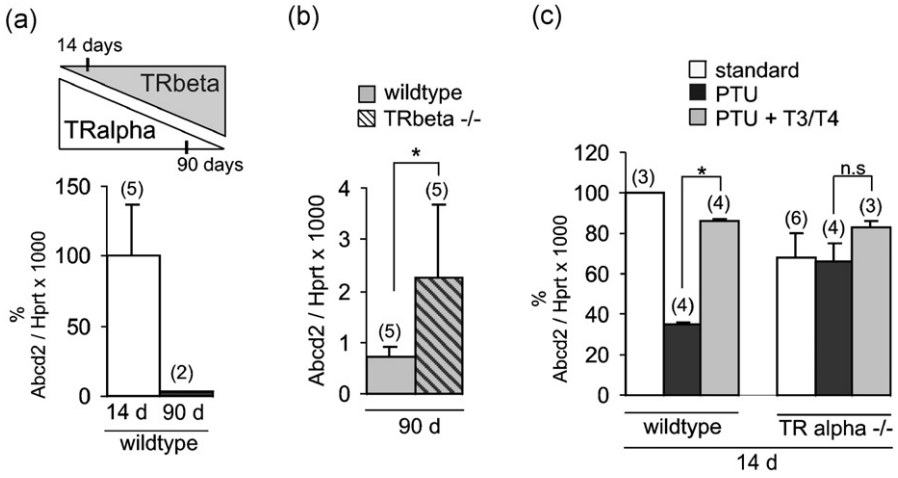

Fig. 6. T_3-dependent modulation of *Abcd2* expression by TRα in the developing and by TRβ in the adult liver of mice. Abcd2 mRNA levels were determined by QRT-PCR in liver isolated from (a) 14-day-old and 90-day-old wild-type, (b) 90-day-old TRβ-deficient and wild-type mice, and (c) 14-day-old TRα-deficient and wild-type mice rendered hypo- or hyperthyroid by treatment with PTU or a combination of PTU and T_3/T_4. mRNA levels were determined in triplicate for each sample and normalized to the number of Hprt mRNA copies. Statistically significant differences by Student's *t*-test are indicated by asterisks ($p < 0.05$).

Assuming that TRs exert an important biological effect on *Abcd2* expression, we investigated whether changes in Abcd2 mRNA levels correlate with absence or presence of the TR isoforms and T_3 abundance. Thus, RNA was isolated from the liver of 14- or 90-day-old wild-type mice, and *Abcd2* expression was evaluated by quantitative real-time RT-PCR (QRT-PCR). High levels of Abcd2 mRNA were found in the liver of young mice, whereas a drastic reduction was observed in adults (Fig. 6a), which would be in good agreement with a stimulatory role of TRα and a repressive effect of TRβ on *Abcd2* expression. To further prove the role of TRβ in this developmental downregulation in older mice, we compared hepatic Abcd2 mRNA levels of 90-day-old TRβ-deficient and wild-type mice. *Abcd2* expression was found to be elevated in the liver of adult mice lacking TRβ compared with age-matched wild-type controls (Fig. 6b). These data support our hypothesis that at low abundance of T_3, TRβ acts as negative regulator of hepatic *Abcd2* expression.

TRα deficiency attenuates T_3 responsiveness of the *Abcd2* gene in the liver of young mice

Next, we explored the contribution of TRα and T_3 to the relatively high level of *Abcd2* expression in the liver of young mice in more detail. Thus, 14-day-old TRα-deficient or wild-type control mice were treated either with PTU, which inhibits the 5′-deiodinase required to convert the T_4 form of thyroid hormone into the bio-active T_3 form, thus resulting in decreased intracellular levels of T_3 (hypothyroid state), or with a combination of PTU and T_3/T_4 in order to induce a hyperthyroid state. In similar treatment paradigms, TR-mediated repression or activation of T_3 target genes has been observed (Plateroti et al., 2006; Poguet et al., 2003), while hepatic SREBP1c mRNA levels remained unaltered (Shin and Osborne, 2003). RNA was isolated from the liver of treated or untreated wild-type and TRα-deficient mice, and Abcd2 mRNA levels were analyzed by QRT-PCR (Fig. 6c). In wild-type mice, a drastic reduction of *Abcd2* expression was observed in the absence of T_3 (PTU-treated) that was restored to pre-treatment levels after administration of T_3/T_4. Thus, in 14-day-old wild-type mice, high levels of Abcd2 mRNA correlate with a high T_3 status, which is in good agreement with our in vitro results. In mice lacking TRα, hepatic Abcd2 mRNA levels remained unaltered after PTU or PTU + T_3/T_4 treatment. In conclusion, these data indicate that the T_3-dependent modulation of *Abcd2* observed in wild-type animals is dependent on TRα.

Discussion

Pharmacological stimulation of the *ABCD2* gene has been suggested as potential therapeutic approach for X-ALD (Kemp et al., 1998), necessitating a detailed characterization of the endogenous *ABCD2* expression and the function of the encoded ALDRP. Intriguingly, one of the key regulators of *ABCD2* transcription seems to be a combined SRE/DR-4 element present both in the human and murine *ABCD2* promoter. Thus, understanding the relationship between the transcriptional activation mediated by SREBP1 and a complex network of DR-4-binding transcription factors, including TRα, TRβ, LXRα, and RXRα, could be crucial for a safe yet

effective modulation of *ABCD2* expression in X-ALD patients. In this context, TRα is of particular interest, as this nuclear receptor is highly abundant in X-ALD target tissues, e.g. brain, adrenal gland and testis (Nishimura et al., 2004).

In this report, we describe a functional interaction of SREBP1 and TRα or TRβ on the human *ABCD2* promoter. In the presence of T_3 ligand, both TRα and TRβ exert a co-stimulatory effect on SREBP1-mediated *ABCD2* expression. In the absence of T_3, however, TRβ but not TRα negatively affects SREBP1-dependent expression. In this context, it is interesting to note that in reporter gene assays, the isolated DR-4 element from the human *ABCD2* promoter region confers strong activation by TRα (cf. Results) or TRβ (Fourcade et al., 2003) alone in a ligand-dependent manner, whereas only a slight, not consistently significant induction was observed in the promoter context (Fourcade et al., 2003). However, in combination with the prominent *ABCD2*-activator SREBP1, both TRα and TRβ exert a co-regulatory function on the native *ABCD2* promoter.

Co-transfection of TRβ and SREBP1 in the absence of T_3 resulted in reduced expression from the *ABCD2* promoter-driven luciferase construct, whereas a derepression was observed in response to T_3. Interestingly, this stimulatory effect was abolished after introduction of DR-4 point mutations that prevent binding of TRβ, or by using a mutated TRβ unable to bind DNA. In contrast, the repressive effect by unliganded TRβ on *ABCD2* expression was independent of these mutations. Thus, two different modulatory effects by TRβ on SREBP1-activated transcription of the *ABCD2* promoter can be discriminated: (i) stimulation that requires binding of TRβ to the SRE/DR-4 sequence and (ii) repression independent of a physical interaction between TRβ and the SRE/DR-4 element or any other DNA motif within the *ABCD2* promoter region. The repressive effect of unliganded TRβ on the *ABCD2* promoter is in agreement with a previously proposed "off-DNA" model for negative control of a subset of TR-regulated genes (Tagami et al., 1997, 1999). According to this model, neither the DNA-binding domain nor direct interaction between TRβ and the DNA of the target gene is required for TR-mediated transrepression, possibly occurring through squelching of cofactors necessary for stimulation of target gene expression. In support of this notion, it can be excluded that unliganded TRβ exerts a generalized direct negative effect on SREBP1, as demonstrated with the Scd1-SRE reporter construct lacking a DR-4. In addition, the repressive effect on SREBP1 stimulation conferred by the TRβ DNA-binding mutant also excludes a model in which TRβ would regulate the expression of a second transcription factor. To our knowledge, the *ABCD2* promoter is the first example of TR-dependent regulation of transcription where both, DNA-dependent and -independent mechanisms operate on the same gene.

Based on the results observed in luciferase assays, a model emerged whereby SREBP1 and TRα concurrently bind the ABCD2 SRE/DR-4 motif. This hypothesis of an oligomeric complex between SREBP1 and TRα was further supported by our EMSA experiments using nuclear extracts. Indeed, two different DNA–protein interactions were observed, a preferentially formed complex A and a weaker complex B. The mobility of complex A was not identical with those resulting from the interactions formed on the control (Scd1) SRE (designated complex C and D), thus excluding that SREBP1 alone binds to the ABCD2 SRE/DR-4 sequence. Moreover, complex A rather mirrored the interaction observed with the Acc probe containing a SRE located in close proximity to a DR-4 element and where interaction of SREBP1 and TRα was previously shown (Yin et al., 2002). In addition, EMSAs with the DR-4mutM2 version of the ABCD2 SRE/DR-4 that is unable to bind SREBP1 provided further evidence for the presence of SREBP1 in complex A. Intriguingly, complex A was also retained with the ABCD2 SRE/DR-4mutM1 mutation that diminished the interaction with the DR-4-binding transcription factors TR/RXRα. This could possibly be explained by the fact that DR-4 mutation M1 did not completely abolish binding of TR/RXRα and thus, in the presence of SREBP1 and other cofactors available in the nuclear extract, formation of complex A could be stabilized. The second DNA–protein interaction observed with the ABCD2 SRE/DR-4, designated complex B, was not consistently present but was favored in the absence of complex A, as also demonstrated with the ABCD2 SRE/DR-4mutM2 probe. In addition, complex B resembled the interaction formed on the Acc DNA probe carrying a DR-4 element but lacking the SRE. Taken together, these data suggest that the SRE/DR-4 sequence is preferentially occupied concurrently by SREBP1 and a DR-4-binding protein, probably TR. When SREBP1 is not available, DR-4-binding proteins alone could interact with the SRE/DR-4, possibly in the form of a TR/RXRα heterodimer.

Our in vitro results showing that ligand-activated TRα and TRβ stimulate or derepress, respectively, SREBP1-dependent induction of the *ABCD2* promoter are supported in vivo by experiments comparing *Abcd2* expression of mice with different TRα/TRβ ratio and T_3 status. In the murine liver, TRβ is drastically upregulated upon maturation to adulthood whereas the levels of TRα and T_3 are reduced (Rodd et al., 1992). Accordingly, 90-day-old mice may reflect a state of "repression by unliganded TRβ" (high TRβ and low T_3 levels). In contrast, younger (14-day-old) animals represent a state of "activation by TRα" with high levels of ligand-bound TRα. In line with these considerations, hepatic Abcd2 mRNA levels were

significantly increased in 14-day-old wild-type mice compared with 90-day-old adults. This is in accordance with a previous study describing a temporal regulation of the *Abcd2* gene in the liver of rats (Albet et al., 2001). Moreover, Abcd2 mRNA levels were elevated in the liver of adult mice lacking TRβ, which is in good agreement with our hypothesis of a TRβ-dependent repression. In this context, the T_3-dependent induction of hepatic Abcd2 mRNA levels in adult mice observed by Fourcade et al. (2003) could be interpreted as a T_3-induced derepression of the *Abcd2* gene mediated by TRβ. Alternatively, because TRβ-deficient mice are congenitally hyperthyroid due to an impairment of the pituitary/thyroid axis (Gauthier et al., 1999) the observed upregulation of *Abcd2* expression in our experiments could also be explained by activation of TRα at very high levels of T_3.

The contribution of T_3 and TRα to high levels of Abcd2 mRNA in the liver of young mice was also investigated using 14-day-old TRα-deficient or wild-type mice. Animals were made either hypo- or hyperthyroid by administration of PTU or a combination of PTU and T_3/T_4, respectively. This treatment paradigm demonstrated that T_3-activated TRα stimulates expression of the *Abcd2* gene in the liver of wild-type mice. This T_3-dependent induction was lost in TRα-deficient animals. The higher level of Abcd2 mRNA in PTU-treated TR$\alpha^{-/-}$ animals compared with wild-type littermates was surprising, but might be caused by additional dysregulation of transcription in mice lacking TRα. With respect to the *Abcd2* promoter these alterations could either lead to more efficient binding of SREBP1 or to occupation of the SRE/DR-4 site by other DR4-binding transcription factors.

To sum up, it appears that both transcription factors (SREBP and TR) have to concurrently bind the SRE/DR-4 element since neither TRα nor TRβ activates the *ABCD2* promoter in the absence of SREBP1, which is also supported by our EMSA experiments using nuclear extracts. Such a direct interaction between SREBP1 and TRs has been described for transcriptional activation of the chicken *Acc* gene where a SRE is located in close proximity to a DR-4 element and interaction of both transcription factors is required for maximal stimulation of the gene.

Pharmacological gene therapy for X-ALD is aimed at upregulating the redundant *ABCD2* gene to compensate for biochemical defects caused by loss of the X-ALD protein. Within the present study, we identify TRs as specific modulators of SREBP1-mediated stimulation of *ABCD2* transcription. Future studies will address, whether novel highly potent cell- or tissue-specific ligands for TRα, TRβ or other DR-4-binding transcription factors that interact with SREBP1 can enhance expression of the *ABCD2* gene in the brain and prevent neurodegeneration in X-ALD.

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