The retinoid X receptor binding to the thyroid hormone receptor: relationship with cofactor binding and transcriptional activity

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Abstract

Transcriptional regulation is mediated by thyroid hormone (tri-iodothyronine, T3) receptors (TR), which bind to T3 response elements as heterodimers with retinoid X receptors (RXR). TR binds to corepressor proteins (CoR) in the absence of T3, which mediate transcriptional repression and to coactivator proteins (CoA) in the presence of T3, which mediate transcriptional stimulation, by recruiting additional proteins to the promoter. To determine the relationship between TR functions and cofactor bindings, we selected 13 single-point mutants on the ligand binding domain of TR, of which T3 bindings were well preserved and created VP16 chimeric receptors. Using mammalian two-hybrid assays, RXR binding in the absence of T3 was almost abolished for Y406K (helix; H10) and L422R (H11), while it was preserved for most other TR mutants. RXR binding was increased for I280K, V284R (H3), and C309K (H6). Addition of T3 enhanced RXR binding and T3 restored the RXR binding to Y406K but not to L422R. CoR binding was reduced for P214R, W219K (H1), R316H (H6), D366R (H9), and M423A (H11) in addition to the mutants of which RXR binding was affected, and CoA binding was impaired for I280K, V284R (H3), C309K (H6), and E457K (H12), indicating that sites for CoR, CoA, and RXR binding partially overlap. CoR binding was well correlated with T3-independent transcriptional regulation and CoA binding was well correlated with T3-dependent regulation, while RXR binding was not correlated with any of these functions among TR mutants, suggesting that transcriptional regulation by TR is mainly mediated by an exchange of CoRs and CoAs.

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Introduction

A subgroup of the nuclear receptor superfamily that includes the receptors for thyroid hormone (TRs), retinoic acid (RARs), and vitamin D are known to form heterodimers with retinoid X receptors (RXR) to modulate the transcription of genes that contain hormone response elements (Lazar et al. 1991, Umesono et al. 1991, Forman et al. 1992). These receptors act as ligand-dependent transcription factors that suppress or stimulate the expression of target genes (Mangelsdorf et al. 1995). Transcriptional stimulation in response to ligand binding of these receptors is mediated by interacting with coactivator proteins (CoAs). These include members of the SRC1 family, CBP/p300, and p/CAF that possess intrinsic histone acetyl transferase (HAT) activity (Bannister & Kouzarides 1996, Chen et al. 1997, Spencer et al. 1997, Blanco et al. 1998), and the DRIP complex (Rachez et al. 1998). TRs also function as potent repressors in the absence of hormone. Unliganded TRs suppress the basal activity of positively regulated promoters by binding to thyroid hormone response elements (TREs; Brent et al. 1989, Damm et al. 1989, Banialhad et al. 1992). Two classes of nuclear corepressors (CoRs), termed nuclear receptor CoR (nCoR; Horlein et al. 1995), and silencing mediator for RARs and TRs (SMRT; Chen & Evans 1995) have been identified, and shown to mediate ligand-independent repression. These CoRs assemble a repression complex that includes Sin3 and histone deacetylases (HDACs) among other proteins (Alland et al. 1997, Heinzel et al. 1997, Nagy et al. 1997). Consequently, transcriptional regulation involves chromatin remodeling caused by histone (de)acetylation by swapping these receptor-assembled HAT and HDAC complexes, in response to ligand binding to TR.

Resistance to thyroid hormone (RTH) is a syndrome of reduced responsiveness of the target tissues to thyroid hormone (tri-iodothyronine, T3; Refetoff et al. 1993). RTH is an autosomal dominant disorder caused by mutations in the TRβ gene. Although the mutant receptors are transcriptionally inactive, they inhibit normal receptor function in a dominant negative manner to cause hormone resistance. We and others
have found that the dominant negative activity correlates well with CoR binding to the RTH mutants and consequently with their silencing activity (Yoh et al. 1997, Tagami & Jameson 1998). Receptor dimerization is also believed to be important to the dominant negative activity (Nagaya & Jameson 1993, Kitajima et al. 1995).

Crystal structures of the ligand binding domains (LBDs) of TRs have revealed that the ligand is completely buried within the hydrophobic core of the domain (Wagner et al. 1995). Upon hormone binding, the carboxy-terminal activation domain (AF-2) forms an amphipathic helix, with its hydrophobic face constituting a part of the hormone-binding cavity. The AF-2 mediates hormone-dependent binding of CoA proteins. Ligand activation of transcription induces the formation of a hydrophobic cleft that is created by folding the carboxy-terminal α helix against a scaffold of three other helices (Feng et al. 1998). An LXXLL motif-containing the α helix from CoAs interacts with a hydrophobic groove within the ligand-bound LBDs (Darimont et al. 1998, Nolte et al. 1998, Shiau et al. 1998). Here, we defined the critical sites for CoRs, CoAs, and the heterodimeric partner, RXR, using a mammalian two-hybrid method. In addition, having established the binding properties to these proteins of the individual mutant TRs, the transcriptional activities were determined allowing analysis of the contribution of CoRs, CoAs, and RXR binding to transcriptional regulation.

**Materials and methods**

**Plasmid constructions**

The mutant hTRβ1 cDNAs were prepared by oligonucleotide-directed mutagenesis and verified by DNA sequencing as described previously (Tagami et al. 1997a). The numbering of the amino acid residues of TRβ is based on a consensus nomenclature (Beck-Peccoz et al. 1994). Mutant and WT receptor cDNAs were subcloned into pCMX (Umesono et al. 1991) and pCMX-VP16 (Tagami et al. 1999) for in vitro transcription/translation and for transient expression in transfected cells. The Gal4-NGO (residues 1552–2453), Gal4-SMRT (residues 876–1495), Gal4-SRC1 (residues 213–1061), Gal4-GRIP1 (residues 480–1462) and Gal4-RXR (LBD) contain the indicated TR interaction domains of these proteins fused to downstream of the Gal4-DBD in-frame in pSG424 (Tagami et al. 1998a,b).

The reporter plasmid TRE-tk-Luc, F2-tk-Luc, DR4-sv40-Luc, and UAS-tk-Luc were described previously (Tagami et al. 1997a,b, 1999). The tk indicates a thymidine kinase promoter and SV40 is a simian virus 40 promoter. The reporter plasmid used for the promoter interference assay, GRE-tk-DR4-Luc, was created by insertions of a DR4-TRE downstream and of a glucocorticoid response element (GRE) upstream of the promoter (Tagami et al. 1998c).

**Transient expression assays**

TSA-201 cells, a clone of human embryonic kidney 293 cells (Tagami et al. 1997a), were grown in DMEM (Nikken Biomedical Lab., Kyoto, Japan) with 10% charcoal-stripped fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) and were transfected by the calcium phosphate method (Tagami et al. 1997a). The total amount of expression plasmid DNA was kept constant in the different experimental groups by adding corresponding amounts of the same plasmids without receptor. After exposure to the calcium phosphate-DNA precipitate for 8 h, DMEM with 10% charcoal-stripped FBS was added. Cells were harvested after 40 h for measurements of luciferase activity, according to the manufacturer’s instructions (Dual-Luciferase Reporter Assay System, Promega). The transfection efficiencies were corrected with the internal control.

**Electrophoretic mobility shift assay**

Mutant, wild-type TRβ or RXRa was transcribed and translated using TNT-coupled reticulocyte lysate system (Promega) labeled with Transcend non-radioactive translation detection system (Promega). Reticulocyte lysates expressing TR (2 μl) with or without RXRa (2 μl) were preincubated at room temperature in a 20 μl reaction with a binding buffer consisting of 20 mM HEPES, pH 7.8, 50 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 50 μg/ml poly(dI-dC) for 15 min. Unlabeled LAP-TREs (sense strand, agtcTGACCTgacgtcAGGTCActcga) or DR4-TREs (sense strand, agtctAGGTCActtAGGTGTActcga) were added, and the mixture was incubated for an additional 20 min. The protein-DNA complexes were analyzed by electrophoresis through 5% polyacrylamide gel containing 2.5% glycerol in 0.5 mM Tris borate, 1 mM EDTA. The proteins were electroblotted onto nylon membranes and detected using the streptavidine alkaline phosphatase substrate. Nuclear extracts (2.5 μg) from transfected TSA-201 cells were preincubated at room temperature in a 20 μl reaction using LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL, USA) with 50 μg/ml poly(dI-dC) for 15 min. Biotin-labeled DR4-TREs were added, and the mixture was incubated for an additional 20 min. The protein-DNA complexes were analyzed by electrophoresis through 5% polyacrylamide gel. The proteins were electroblotted onto nylon membranes.
and detected using the streptavidine-HRP conjugate and the chemiluminescence substrate. For supershift assays, anti-TRβ1 (Lin et al. 1991) or anti-RXRα antibodies (Perseus Proteomics Inc., Tokyo, Japan) were added, and the mixture was preincubated at 4°C overnight.

**Western blotting**

Nuclear extracts (10 μg) from transfected TSA-201 cells prepared using Nuclear Extract kit (Active Motif, Carlsbad, CA, USA) were analyzed by SDS-PAGE with 10% acrylamide gel. The proteins were electroblotted onto nitrocellulose membranes, followed by reaction with monoclonal antibodies (J52) against the amino-terminal domain of TRβ1 (Lin et al. 1991), as described previously (Tagami et al. 1993).

**Statistical analysis**

Data were analyzed by ANOVA with post hoc Dunnett’s tests to compare with the control. Pearson’s correlation coefficients were determined by using regression analysis.

**Results**

**DNA binding of TR mutants**

Among more than 100 different TR mutants that were created based on the TR LBD X-ray crystallographic

**Figure 1** EMSA, western blotting, and promoter interference assays. (A) Mutant, wild-type TRβ or RXRα was transcribed and translated using a reticulocyte lysate system labeled with a nonradioactive translation detection system, and analyzed by the SDS-PAGE with 10% acrylamide gel (top). Reticulocyte lysates expressing TR (2 μl) with or without RXRα (2 μl) were incubated with unlabeled LAP-TREs or DR4-TREs. The protein-DNA complexes were analyzed by EMSA. The positions of the TR/TR homodimer (homo), TR heterodimer with RXR (hetero) are indicated. (B) Nuclear extracts (10 μg) from transfected TSA-201 cells were analyzed by SDS-PAGE and were electroblotted onto nitrocellulose membranes, followed by reaction with J52-mAbs. Middle: EMSA was performed using nuclear extracts (2-5 μg) of transfected TSA-201 cells. The DNA binding site is biotin-labeled DR4-TRE. Bottom: Supershift assays were performed using anti-RXRα or -TRβ1 antibodies. In two lanes at the right end, T3 (1 μM) were added into the reaction mixture. (C) The principle of the promoter interference assay is shown. A DR4-TRE is inserted downstream of TATA box of GRE-tk promoter. Binding of TR blocks basal transcription of the construct, which is stimulated by glucocorticoid response elements upstream of the promoter. TR expression plasmids (50 ng) for the indicated mutants were transfected together with 100 ng of GRE-tk-DR4-Luc in the absence or presence of T3. Results are the mean ± S.D. from at least three transfections performed in triplicate.

To test the DNA binding activity of the TR mutants, EMSA was performed. First, the TR protein expressed using a reticulocyte lysate system was incubated with LAP or DR4 oligonucleotides (Fig. 1A). The homodimerization and heterodimerization with RXR was variously observed among mutants. In general, homodimerization was impaired in W219K, R316H, D366R, and M423A and almost abolished in P214R, Y406K, and L422R. The heterodimerization with RXR was markedly impaired in P214R, Y406K, and L422R.

Next, transient expression experiments were performed using TSA201 cells, which are derivatives of 293 cells. To test the protein expression of the TR mutants in the nucleus, we performed western blotting using anti-TR antibody (Lin et al. 1991). The protein expression level of each mutant in the nuclear fraction of transfected cells was confirmed (Fig. 1B, top). The nuclear fraction of transfected cells was incubated with DR4 oligonucleotides. As shown in the middle panel of Fig. 1B, the DNA binding was impaired in W219K, D366R, and E457K, and almost abolished in P214R, Y406K, and L422R. To clarify whether the DNA binding is homodimer or heterodimer with endogenous RXR, supershift assays were performed using anti-RXR or -TR antibodies (Fig. 1B, bottom). Incubation with anti-RXR antibodies decreased the intensity of the DNA binding and generated a slower migrating band, indicating that the DNA binding contains endogenous RXR. Because T₃ dissociates TR homodimers (Anderson et al. 1992, Yen et al. 1992), T₃ was added into the incubation. The complex was not decreased by T₃ (Fig. 1B, bottom, and the two lanes at right ends). Finally, we utilized a promoter interference assay. A DR4-TRE was inserted downstream of the site of

Figure 2 Interactions of RXR with mutant TRs in a mammalian two-hybrid assay. (A) The format of the mammalian two-hybrid experiment is shown. (B) VP16-TR expression plasmids (20 ng) for the indicated mutants were cotransfected into TSA-201 cells together with the Gal4-responsive reporter gene, UAS-tk-Luc (50 ng), and 20 ng of Gal4-RXR in the absence or presence of 100 nM T₃. Results are the mean ± S.D. from at least three transfections performed in triplicate. *P < 0.01 versus WT in the absence of T₃. †P < 0.01 versus WT in the presence of 100 nM T₃. C, top: the indicated TR or RXR was transcribed and translated using a reticulocyte lysate system labeled with a non-radioactive translation detection system. Reticulocyte lysates expressing TR (2 μl) and RXR (2 μl) in the presence or absence of T₃ (1 μM) were incubated with unlabeled LAP-TREs and the protein-DNA complexes were analyzed by EMSA. The positions of the TR/TR homodimer (homo), TR heterodimer with RXR (hetero) are indicated. Bottom: VP16-TR expression plasmids (20 ng) for the indicated mutants were cotransfected into TSA-201 cells together with the Gal4-responsive reporter gene, UAS-tk-Luc (50 ng), and 20 ng of Gal4-SRC1 or Gal4-RXR in the absence or presence of 100 nM T₃. Results are the mean ± S.D. in a log scale from at least three transfections performed in triplicate.
transcriptional initiation of tk promoter, which is stimulated by glucocorticoid, resulting in transcriptional inhibition when receptors were bound to this element, as shown in Fig. 1C. This assay therefore represents TR interaction with DNA in cells (Tagami et al. 1998c). DNA binding with DR4-TRE was relatively well preserved for all the mutants, except for Y406K and L422R.

**RXR binding of TR mutants**

Using a mammalian two-hybrid assay, the RXR binding was examined among these mutants. The format of assay is shown in Fig. 2A. The RXR-LBD was fused to the DBD of the yeast transcription factor, Gal4. The full-length of each TR mutant was fused to the

![Diagram A](image1.png)

**Figure 3** DNA binding in one-hybrid assays. (A) The format of the mammalian one-hybrid experiment is shown. (B–D) VP16-TR expression plasmids (20 ng) for the indicated mutants were cotransfected into TSA-201 cells together with indicated T3-responsive reporter genes, TRE-tk-Luc (50 ng), F2-tk-Luc (100 ng) or DR4-sv40-Luc (10 ng) in the absence or presence of 100 nM T3. Results are the mean ± S.D. from at least three transfections performed in triplicate. *P < 0.01 versus corresponding WT in the absence of T3. †P < 0.01 versus corresponding WT in the presence of 100 nM T3. (E and F) The luciferase activity reflecting RXR binding (Fig. 2B) is plotted versus the luciferase activities reflecting the VP16-TR binding to TREs (B–D) in the absence (E) or presence of T3 (F).
transcriptional activation domain of VP16 to detect interactions between the Gal4 fusion proteins and the TRs. Gal4-RXR and VP16-TR mutants were transfected with a Gal4 reporter gene, UAS-tk-Luc. The RXR binding, in the absence of T3, was almost abolished for Y406K in helix 10 and L422R in helix 11, and slightly impaired for P214R, V319K, and E457K, while well preserved for most of the other TR mutants (Fig. 2B). Particularly, the RXR binding was rather increased for I280K, V284R in helix 3 and C309K in helix 6. The addition of T3 enhanced the RXR binding proportionally to the RXR binding in the absence of T3, except for L422R. Interestingly, T3 restored the RXR binding to Y406K TR mutant. This phenomenon was confirmed by the EMSA (Fig. 2C, top). This may be due to conformational changes induced by T3 binding but not due to stabilization by subsequent CoA binding because the Y406K/E457K composite mutant disclosed little or no SRC binding but preserved the T3-induced RXR binding (Fig. 2C, bottom); the CoA binding of the E457K TR mutant is impaired without involvement of T3 binding (see Fig. 5).

The in vivo DNA binding was also examined using VP16-full-length TR mutants to palindromes (TREpal), inverted palindromes (F2/IP-6) and direct repeats (DR-4) type TREs (Fig. 3A–D). The RXR binding was well correlated with the DNA binding in cells to these TREs in the absence of T3 (Fig. 3E), probably because cells contain sufficient endogenous RXR or TR auxiliary proteins (Tagami et al. 1997b). In the presence of T3 (Fig. 3F), although a correlation between RXR binding and TRE binding in cells was also observed, the correlation disappears if points for the vector (control) are excluded.

CoR binding and CoA binding of TR mutants

Next, we examined CoR binding using Gal4-NCoR or Gal4-SMRT in a similar fashion. Gal4-NCoR or Gal4-SMRT was cotransfected with VP16-full-length TR

Figure 4 Interactions of CoRs with mutant TRs in mammalian two-hybrid assays. (A and B) VP16-TR expression plasmids (20 ng) for the indicated mutants were cotransfected into TSA-201 cells together with the Gal4-responsive reporter gene, UAS-tk-Luc (50 ng), and 20 ng of Gal4-NCoR (A) or Gal4-SMRT (B) in the absence or presence of 100 nM T3. Results are the mean ± S.D. from at least three transfections performed in triplicate. *P < 0.01 versus corresponding WT in the absence of T3. †P < 0.01 versus corresponding WT in the presence of 100 nM T3. (C) The luciferase activity reflecting NCoR binding (A) is plotted versus the luciferase activities reflecting SMRT binding (B) in the absence of T3. (D) The luciferase activity reflecting RXR binding (Fig. 2B) is plotted versus the luciferase activities reflecting CoR binding (A and B) in the absence of T3.

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mutants (Fig. 4A and B). The binding abilities to NCoR, in the absence of T3, were moderately impaired for R316H and M423A, and markedly reduced for P214R, W219K in helix 1, D366R in helix 9 in addition to I280K, V284R, and C309K (up-mutants for RXR binding), and Y406K and L422R (down-mutants for RXR binding). The results using Gal4-SMRT were almost identical to the NCoR binding and a strong correlation was obtained between the SMRT binding and the NCoR binding among TR mutants (Fig. 4C), but the pattern of RXR binding was different from that of CoR binding (Fig. 4D).

Similarly, the CoA interactions with these TR mutants were examined. Gal4-SRC1 or Gal4-GRIP1 was cotransfected with VP16-full length TR mutants (Fig. 5A and B). The binding abilities to SRC1, in the presence of T3, were moderately reduced for C309K and R316H in helix 6, and markedly impaired for I280K, V284R in helix 3, and E457K in helix 12 TR mutants of which T3 bindings were preserved. The results using Gal4-GRIP1 were almost identical to the SRC1 binding and a strong correlation was obtained between the SRC1 binding and the GRIP1 binding among TR mutants (Fig. 5C). However, the pattern of RXR binding among TR mutants was different from that of CoA binding (Fig. 5D).

Transcriptional activities of mutant receptors

Having established the cofactor binding of the mutant TRs, the transcriptional activities of these mutants were examined. Positively regulated reporter genes, TREpal-tk-Luc, F2-tk-Luc and DR4-sv40-Luc, and a negatively regulated reporter gene, TSHα-Luc, were used to characterize the transcriptional activities of the mutants in the absence or presence of T3. In the absence of T3, although the silencing activity on the positively regulated reporters and the basal stimulation on the TSHα promoter by unliganded TR of E227R, R316H, V319K, M423A, and E457K were well preserved, those of other mutants were impaired to various degrees (Fig. 6A–D). Overall, there was a good correlation was obtained between the SRC1 binding and the GRIP1 binding among TR mutants (Fig. 5C). However, the pattern of RXR binding among TR mutants was different from that of CoA binding (Fig. 5D).
correlation between the silencing activities on the positively regulated reporters and the basal stimulation on the TSHz promoter among mutants (Fig. 7A). The silencing activities on TREs and the basal stimulation of TSHz were also compared with the CoR binding, revealing a significant positive correlation between these activities and the CoR binding (Fig. 7B). By contrast, when the silencing activities on TREs and the basal stimulation of TSHz were plotted against the RXR binding, a correlation was not observed (Fig. 7C). There was a negative correlation, if anything, mainly because increased (I280K, V284R, and C309K) RXR binding mutants showed impaired silencing on TREs and basal stimulation of TSHz.

In the presence of T3, T3-dependent stimulation on the positively regulated reporters and T3-dependent repression on the TSHz promoter were moderately impaired for C309K and R316H, and markedly reduced for I280K, V284R, and E457K (Fig. 8A–D). There was a good correlation between the T3-dependent stimulation on the positively regulated reporters and the T3-dependent repression on the TSHz promoter among mutants (Fig. 9A). The T3-dependent stimulation on TREs and T3-dependent repression on the TSHz were significantly correlated with TR-CoA binding (Fig. 9B). Again, a negative correlation was observed with TR-RXR binding (Fig. 9C), mainly because increased (I280K and V284R) RXR binding mutants showed impaired T3-dependent activation on TREs and inhibition of TSHz.

Discussion

Based on the X-ray crystal structure of the TR and other nuclear receptors, the AF-2 domain in helix 12 of the LBD has been proposed to undergo induced conformational changes after binding to the ligand (Bourguet et al. 1995, Renaud et al. 1995, Wagner et al. 1995). The apposition of helix 12 with helices 3, 5, and 6,
which create a scaffold in the receptor, creates a small hydrophobic cleft that has recently been shown to be a binding site for transcriptional CoAs (Feng et al. 1998). This cleft is bordered by helix 3 (I280, V284, K288), helix 5 (I302, L305, K306), helix 6 (C309), and residues L454, E457, and V458 in helix 12. It has been postulated that this receptor region interacts with the conserved hydrophobic LXXLL motif that is found in several different CoAs (LeDouarin et al. 1995, Heery et al. 1997, Torchia et al. 1997, Feng et al. 1998). The critical region for CoR binding has been illustrated more recently. Initially, it was reported that mutations within the hinge region, or CoR box, disrupt CoR binding (Chen & Evans 1995, Horlein et al. 1995). Subsequently, using a glutathione S-transferase (GST) pull-down assay, Perissi et al. (1999) suggested that some residues in helices 3, 5, and 6 in TRβ, which are important for SRC1 binding, are also involved in N-CoR binding. Nagy et al. (1999) showed that V284, K288, F293, Q301, and L305 in helices 3–6 are critical for SMRT binding. The conserved hydrophobic L/IXXII motif in NCoR and SMRT was also found to interact with TR (Hu & Lazar 1999). Recently, we developed a variety of TR LBD mutants and examined the effects of these mutations on NCoR binding using a GST pull-down assay (Marimuthu et al. 2002). There were three sites of mutational sensitivity. Site 1 (helices 3 and 5) contacts NCoR more directly, and partially overlaps the surface defined for CoA binding but extends underneath where helix 12 rests in the ligand-bound conformation. Site 2 is separated from site 1 by helices 1 and 3 and runs along the side of helix 1. Site 3 is separated from site 1 by the turn between helices 9 and 10 and extends between helices 10 and 11. Sites 2 and 3 form weaker contacts to NCoR, suggesting that they function to define and maintain the presentation and stability of the unliganded TR conformation required for NCoR binding (Marimuthu et al. 2002). The results
in this study not only confirm these findings using different methods but also defined the surface for SMRT binding, which appears to correspond to the surface for NCoR binding.

TR binds to TREs as heterodimers with RXR. The surface of TR for RXR binding has also been studied. Some mutations in helices 10 and 11 disrupted RXR binding in in vitro assays (Ribeiro et al. 2001). The conserved ninth C-terminal heptad in TRs and RARs was initially suggested to be important to form heterodimers (Au-Fliegner et al. 1993, Nagaya & Jameson 1993). Mutations of the leucines in helices 10 and 11 disrupt heterodimerization with RXR. The interaction with RXR was rather enhanced by the mutations in helix 3 (I280K and V284R) and in helix 6 (C309K), suggesting that these helices are also involved with RXR interaction. The possibility that apparent increased RXR binding of these mutants may be due to their decreased CoR binding in our study is unlikely, because the RXR binding of these mutants was still stronger in the presence of T3, in which the effect of CoR binding can be ignored. Consequently, because helices 3 and 6 are shared as the binding sites among CoR, CoA, and RXR, mutations that disrupt CoR and CoA bindings may have rather enhanced RXR binding. Alternatively, CoR and CoA bindings may usually take precedence over RXR binding, with regards to helices 3 and 6.

Transcriptional silencing activity correlates well with the dominant negative activity of TR mutants that are found in patients with the syndrome of RTH (Yoh et al. 1997, Tagami & Jameson 1998). Before the discovery of CoRs, RXR interaction was thought to be necessary for dominant negative inhibition (Nagaya & Jameson 1993). We reported before that the dominant negative activity of the RTH mutants correlates well with both the silencing activity and CoR binding (Tagami & Jameson 1998). We tried to verify which is more responsible for the ability to suppress gene transcription, the interaction with CoRs or that of RXR. By comparing the relationship between CoR binding and silencing activity with the one between RXR binding and silencing, we observed that contribution to silencing is reflected

Figure 8 Function of mutant TRs with respect to positively or negatively regulated reporter genes in the presence of T3. (A–C) The T3-dependent stimulation of positively regulated reporter genes by TR mutants. TR expression plasmids (10 ng) for the indicated mutants were transfected into TSA-201 cells together with TREtk-Luc (50 ng), F2tk-Luc (100 ng) or DR4-sv40-Luc (10 ng) in the absence or presence of T3. (D) The T3-dependent repression of a negatively regulated reporter gene by TR mutants. TR expression plasmids (50 ng) for the indicated mutants were transfected into TSA-201 cells together with TSHα-Luc (100 ng) in the absence or presence of T3. Results are the mean ± s.d. from at least three transfections performed in triplicate. †P<0.01 versus corresponding WT in the presence of 100 nM T3.

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more directly by CoR binding than by RXR binding. CoRs are also involved in the basal activation of negatively regulated genes by unliganded TRs (Tagami et al. 1997a, 1999). Here, we showed that not only the silencing on positively regulated promoters but also the basal activation on a negatively regulated promoter were associated with CoR binding but not with RXR binding properties.

**Figure 9** The relationship of CoA or RXR binding with functions of mutant TRs in the presence of T₃. (A) The T₃-dependent inhibition of a negatively regulated reporter gene (Fig. 8D), expressed as relative inhibition to TRβ₁WT (=100), is plotted versus the T₃-dependent stimulation of positively regulated reporter genes (Fig. 8A–C), expressed as relative stimulation to TRβ₁WT (=100), in the presence of T₃. (B) The CoA binding (Fig. 5A and B) is plotted versus the T₃-dependent stimulation of positively regulated reporter genes (relative stimulation to TRβ₁WT) and the T₃-dependent inhibition of a negatively regulated reporter gene (relative inhibition to TRβ₁WT), in the presence of T₃. (C) The RXR binding (Fig. 2B) is plotted versus the T₃-dependent stimulation of positively regulated reporter genes (relative stimulation to TRβ₁WT) and the T₃-dependent inhibition of a negatively regulated reporter gene (relative inhibition to TRβ₁WT), in the presence of T₃.

**Table 1** Summary of properties of mutant receptors in the absence of tri-iodothyronine (T₃)

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The properties of the mutant TRs were summarized in Tables 1 and 2. The residues that showed impaired interaction with CoRs and RXR are co-localized with the residues that bind to CoAs (Feng et al. 1998). This region on the TRb-LBD structure is depicted as a ribbon diagram based upon the X-ray crystal structure of TRa (Wagner et al. 1995) in Fig. 10. The sites for SMRT binding were almost identical to those for NCoR binding (Marimuthu et al. 2002). The LXXLL motif in the CoA proteins and the L/IXXII motif in the CoR proteins are proposed to interact with nuclear receptors (Heery et al. 1997, Webb et al. 2000). Helices 3 and 5 in TR seem to be important both for the LXXLL and IXXII interactions. By contrast, the AF2 in helix 12 plays a distinct role in TR interactions with CoA and CoR. The AF2 domain makes contact with the LXXLL of CoAs but may have an inhibitory effect on the CoR interaction, since deletion or mutation of the AF2 domain potentiated the CoR interaction with TR (Tagami & Jameson 1998, Pissios et al. 2000) or RXR (Nagy et al. 1999). On the other hand, mutations in the helices 3, 6, 10, and 11 altered interactions with both CoRs and RXR. Since RXR can restore TR and CoR interaction (Zhang et al. 1997), RXR and CoR may create a tertiary complex on this region of TR. Alternatively, one of three L/IXXII motifs of CoR protein may anchor on the helix 1 of TR LBD and the contact by the others may be stabilized by RXR on helices 3–6 and 10–11. Ligand binding recruits the AF2 domain in helix 12 to helices 3–6, to release CoR and form a CoA binding cleft.

In conclusion, we determined the sites for CoRs, CoAs, and the heterodimeric partner, RXR and found that the T3-independent activity and T3-dependent activity correlate very well with the CoR binding and with the CoA binding respectively, but these do not correlate with the RXR binding to TRs.

Table 2 Summary of properties of mutant receptors in the presence of tri-iodothyronine (T3)

<table>
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<tr>
<th>Receptor</th>
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<th>Binding with CoAs</th>
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![Figure 10](https://example.com/figure10.png)

Figure 10 Three-dimensional model of the TR ligand-binding domain. The structure of the ligand-binding domain of TRb is shown as a ribbon diagram based upon the X-ray crystal structure of TRa. The locations of various helices and TRb mutants are shown. The regions for CoR, CoA, and RXR binding are circled.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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