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

Tetsuya Tagami, Hiroyuki Yamamoto, Kenji Moriyama, Kuniko Sawai, Takeshi Usui, Akira Shimatsu and Mitsuhide Naruse

Department of Endocrinology and Metabolism, Clinical Research Institute, Kyoto Medical Center, National Hospital Organization, 1-1 Mukaihata-cho, Fukakusa, Fushimi-ku, Kyoto 612-8555, Japan

(Correspondence should be addressed to T Tagami; Email: ttagami@kyotolan.hosp.go.jp)

Abstract

Transcriptional regulation is mediated by thyroid hormone (tri-iodothyronine, T_3) receptors (TR), which bind to T_3 response elements as heterodimers with retinoid X receptors (RXR). TR binds to corepressor proteins (CoR) in the absence of T_3 , which mediate transcriptional repression and to coactivator proteins (CoA) in the presence of T_3 , 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 T_3 bindings were well preserved and created VP16 chimeric receptors. Using mammalian two-hybrid assays, RXR binding in the absence of T_3 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 T_3 enhanced RXR binding and T_3 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 T_3 -independent transcriptional regulation and CoA binding was well correlated with T_3 -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.

Journal of Molecular Endocrinology (2009) **42**, 415–428

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, Baniahmad *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, T_3 ; Refetoff *et al.* 1993). RTH is an autosomal dominant disorder caused by mutations in the TR β 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 hTR β 1 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 TR β 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-NCoR (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 μ g/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 TR β or RXR α 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 μ l) with or without RXR α (2 μ l) were preincubated at room temperature in a 20 μ l 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 μ g/ml poly(dI-dC) for 15 min. Unlabeled LAP-TREs (sense strand, agtcTGACCTgacgtcAGGTCActcga) or DR4-TREs (sense strand, agcttcAGGTCActtcAGGTCActcga) 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 $\times$ TBE (45 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 μ g) from transfected TSA-201 cells were preincubated at room temperature in a 20 μ l reaction using LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL, USA) with 50 μ g/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 a 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-TR β 1 (Lin *et al.* 1991) or anti-RXR α antibodies (Perseus Proteomics Inc., Tokyo, Japan) were added, and the mixture was pre-incubated at 4 °C overnight.

Western blotting

Nuclear extracts (10 μ g) 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 TR β 1 (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 TR β or RXR α was transcribed and translated using a reticulocyte lysate system labeled with a non-radioactive translation detection system, and analyzed by the SDS-PAGE with 10% acrylamide gel (*top*). Reticulocyte lysates expressing TR (2 μ l) with or without RXR α (2 μ l) 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 μ g) 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 μ g) of transfected TSA-201 cells. The DNA binding site is biotin-labeled DR4-TRE. *Bottom*: Supershift assays were performed using anti-RXR α or -TR β 1 antibodies. In two lanes at the right end, T₃ (1 μ M) 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 $\pm$ s.d. from at least three transfections performed in triplicate.

structure (Wagner *et al.* 1995, Feng *et al.* 1998, Marimuthu *et al.* 2002), we have selected 13 representative TR mutants on the surface of TR LBD, of which T_3 bindings were well preserved (Kitajima *et al.* 1995,

Tagami *et al.* 1997a, Feng *et al.* 1998, Tagami & Jameson 1998, Ribeiro *et al.* 2001, Marimuthu *et al.* 2002).

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 α antibodies decreased the intensity of the DNA binding and generated a slower migrating band, indicating that the DNA binding contains endogenous RXR. Because T_3 dissociates TR homodimers (Anderson *et al.* 1992, Yen *et al.* 1992), T_3 was added into the incubation. The complex was not decreased by T_3 (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 T_3 . Results are the mean $\pm$ s.d. from at least three transfections performed in triplicate. * $P < 0.01$ versus WT in the absence of T_3 . # $P < 0.01$ versus WT in the presence of 100 nM T_3 . C, *top*: the indicated TR or RXR α was transcribed and translated using a reticulocyte lysate system labeled with a non-radioactive translation detection system. Reticulocyte lysates expressing TR (2 μ l) and RXR α (2 μ l) in the presence or absence of T_3 (1 μ M) 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 T_3 . Results are the mean $\pm$ 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

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 T_3 -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 T_3 . Results are the mean $\pm$ s.d. from at least three transfections performed in triplicate. * $P < 0.01$ versus corresponding WT in the absence of T_3 . † $P < 0.01$ versus corresponding WT in the presence of 100 nM T_3 . (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 T_3 (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 T_3 , 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 T_3 enhanced the RXR binding proportionally to the RXR binding in the absence of T_3 , except for L422R. Interestingly, T_3 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 T_3 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 T_3 -induced RXR binding (Fig. 2C, bottom); the CoA binding of the

E457K TR mutant is impaired without involvement of T_3 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 T_3 (Fig. 3E), probably because cells contain sufficient endogenous RXR or TR auxiliary proteins (Tagami *et al.* 1997b). In the presence of T_3 (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 T_3 . Results are the mean $\pm$ S.D. from at least three transfections performed in triplicate. * $P < 0.01$ versus corresponding WT in the absence of T_3 . † $P < 0.01$ versus corresponding WT in the presence of 100 nM T_3 . (C) The luciferase activity reflecting NCoR binding (A) is plotted versus the luciferase activities reflecting SMRT binding (B) in the absence of T_3 . (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 T_3 .

mutants (Fig. 4A and B). The binding abilities to NCoR, in the absence of T_3 , 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 T_3 , 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 T_3 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, TSH α -Luc, were used to characterize the transcriptional activities of the mutants in the absence or presence of T_3 . In the absence of T_3 , although the silencing activity on the positively regulated reporters and the basal stimulation on the TSH α 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

Figure 5 Interactions of CoAs 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-SRC1 (A) or Gal4-GRIP1 (B) in the absence or presence of 100 nM T_3 . Results are the mean $\pm$ s.d. from at least three transfections performed in triplicate. $^{\dagger}P < 0.01$ versus corresponding WT in the presence of 100 nM T_3 . (C) The luciferase activity reflecting SRC1 binding (A) is plotted versus the luciferase activities reflecting GRIP1 binding (B) in the presence of T_3 . (D) The luciferase activity reflecting RXR binding (Fig. 2B) is plotted versus the luciferase activities reflecting CoA binding (A and B) in the presence of T_3 .

correlation between the silencing activities on the positively regulated reporters and the basal stimulation on the TSH α promoter among mutants (Fig. 7A). The silencing activities on TREs and the basal stimulation of TSH α 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 TSH α 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 TSH α .

In the presence of T₃, T₃-dependent stimulation on the positively regulated reporters and T₃-dependent repression on the TSH α 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 TSH α promoter among mutants (Fig. 9A). The T₃-dependent stimulation on TREs and T₃-dependent repression on the TSH α 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 TSH α .

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,

Figure 6 Function of mutant TRs with respect to positively or negatively regulated reporter genes in the absence of T₃. (A–C) The silencing 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 TRE-tk-Luc (50 ng), F2-tk-Luc (100 ng) or DR4-sv40-Luc (10 ng) in the absence of T₃. (D) The basal activation 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 TSH α -Luc (100 ng) in the absence of T₃. Results are the mean $\pm$ s.d. from at least three transfections performed in triplicate. **P* < 0.01 versus corresponding WT in the absence of T₃.

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 TR β , 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

Figure 7 The relationship of CoR or RXR binding with functions of mutant TRs in the absence of T_3 . (A) The basal activation of a negatively regulated reporter gene (Fig. 6D), expressed as relative activation to TR β 1WT (= 100), is plotted versus the silencing of positively regulated reporter genes (Fig. 6A–C), expressed as relative repression to TR β 1WT (= 100), in the absence of T_3 . (B) The CoR binding (Fig. 4A and B) is plotted versus the silencing of positively regulated reporter genes (relative repression to TR β 1WT) and the basal activation of a negatively regulated reporter gene (relative activation to TR β 1WT) in the absence of T_3 . (C) The RXR binding (Fig. 2B) is plotted versus the silencing of positively regulated reporter genes (relative repression to TR β 1WT) and the basal activation of a negatively regulated reporter gene (relative activation to TR β 1WT) in the absence of T_3 .

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 T_3 , 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 T_3 . (A–C) The T_3 -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 TRE-tk-Luc (50 ng), F2-tk-Luc (100 ng) or DR4-sv40-Luc (10 ng) in the absence or presence of T_3 . (D) The T_3 -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 T_3 . Results are the mean $\pm$ s.d. from at least three transfections performed in triplicate. † $P < 0.01$ versus corresponding WT in the presence of 100 nM T_3 .

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.

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.

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

	Homo-dimer	Hetero-dimer	Binding with NE	Promoter interference	Binding with RXR	Binding with TREs	Binding with CoRs	Silencing	Basal activation
Receptor									
Figure no.	1A	1A	1B	1C	2B	3B–D	4A–B	6A–C	6D
WT	+	+	+	++	+	+	+	+	+
P214R	–	–	–	+	±	+	±	±	±
W219K	±	+	±	+	+	+	±	±	±
E227R	++	+	+	++	+	+	+	+	+
I280K	++	++	+	+	++	++	–	–	–
V284R	++	++	+	+	++	++	–	–	±
C309K	+	+	+	+	++	++	–	–	–
R316H	±	+	+	+	+	+	±	+	+
V319K	++	++	+	++	±	+	+	+	+
D366R	±	+	±	+	+	+	±	±	–
Y406K	–	–	–	±	–	–	±	–	–
L422R	–	–	–	–	–	–	±	±	±
M423A	±	++	+	+	+	+	±	+	+
E457K	+	+	±	++	±	±	+	+	+

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

	Binding with RXR	Binding with TREs	Binding with CoAs	Stimulation	Repression
Receptor Figure no.	2B	3B–D	5A–B	8A–C	8D
WT	+	+	+	+	+
P214R	+	+	+	+	+
W219K	+	+	+	+	+
E227R	+	+	+	+	+
I280K	+	+	–	–	–
V284R	++	+	–	–	–
C309K	++	+	±	±	–
R316H	+	+	±	±	±
V319K	+	+	+	+	+
D366R	+	+	+	+	+
Y406K	+	+	+	+	–
L422R	–	±	+	+	+
M423A	+	+	+	+	+
E457K	+	+	–	–	±

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 TR β -LBD structure is depicted as a ribbon diagram based upon the X-ray crystal structure of TR α (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.

Figure 10 Three-dimensional model of the TR ligand-binding domain. The structure of the ligand-binding domain of TR β is shown as a ribbon diagram based upon the X-ray crystal structure of TR α . The locations of various helices and TR β 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.

Funding

This study was supported in part by the grants from The Japanese Ministry of Education and Science (No.11671103 and No.17590973 to TT).

Acknowledgements

The authors are grateful to Drs Adhirai Marimuthu, Brian L West, John D Baxter and J Larry Jameson for kindly providing mutant TRs and Drs S-Y Cheng and F Furuya for the mAbs of TR β . We also thank Ms Keiko Matsuda, Ms Kumiko Kushii, and Ms Yoshiko Sakaguchi for their excellent secretarial assistance.

References

- Alland L, Muhle R, Hou H Jr, Potes J, Chin L, Schreiber-Agus N & DePinho RA 1997 Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. *Nature* **387** 49–55.
- Anderson ML, Nordstorm K, Demczuk S, Harbes M & Vennstrom B 1992 Thyroid hormone alters the DNA binding properties of chicken thyroid hormone receptors α and β . *Nucleic Acids Research* **20** 4803–4810.
- Au-Fliegner M, Helmer E, Casanova J, Raaka BM & Samuels HH 1993 The conserved ninth C-terminal heptad in thyroid hormone and retinoic acid receptors mediates diverse responses by affecting heterodimer but not homodimer formation. *Molecular and Cellular Biology* **13** 5725–5737.
- Baniahmad A, Kohne AC & Renkawitz R 1992 A transferable silencing domain is present in the thyroid hormone receptor, in the v-erbA oncogene product and in the retinoic acid receptor. *EMBO Journal* **11** 1015–1023.
- Bannister A & Kouzarides T 1996 The CBP co-activator is a histone acetyltransferase. *Nature* **384** 641–643.
- Beck-Peccoz P, Chatterjee VK, Chin WW, DeGroot LJ, Jameson JL, Nakamura H, Refetoff S, Usala SJ & Weintraub BD 1994 Nomenclature of thyroid hormone receptor beta-gene mutations in resistance to thyroid hormone: consensus statement from the first workshop on thyroid hormone resistance. *Journal of Clinical Endocrinology and Metabolism* **78** 990–993.
- Blanco JC, Minucci S, Lu J, Yang XJ, Walker KK, Chen H, Evans RM, Nakatani Y & Ozato K 1998 The histone acetylase PCAF is a nuclear receptor coactivator. *Genes and Development* **12** 1638–1651.
- Bourguet W, Ruff M, Chambon P, Gronemeyer H & Moras D 1995 Crystal structure of the ligand-binding domain of the human nuclear receptor RXR- α . *Nature* **375** 377–382.
- Brent GA, Dunn MK, Harney JW, Gulick T, Larsen PR & Moore DD 1989 Thyroid hormone aporeceptor represses T₃-inducible promoters and blocks activity of the retinoic acid receptor. *New Biologist* **1** 329–336.
- Chen JD & Evans RM 1995 A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* **377** 454–457.
- Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, Privalsky ML, Nakatani Y & Evans RM 1997 Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimetric activation complex with P/CAF and CBP/p300. *Cell* **90** 569–580.
- Damm K, Thompson CC & Evans RM 1989 Protein encoded by v-erbA functions as a thyroid-hormone receptor antagonist. *Nature* **339** 593–597.
- Darimont BD, Wagner RL, Apriletti JW, Stallcup MR, Kushner PJ, Baxter JD, Fletterick RJ & Yamamoto KR 1998 Structure and specificity of nuclear receptor-coactivator interactions. *Genes and Development* **12** 3343–3356.
- Feng W, Ribeiro RC, Wagner RL, Nguyen H, Apriletti JW, Fletterick RJ, Baxter JD, Kushner PJ & West BL 1998 Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. *Science* **280** 1747–1749.
- Forman BM, Casanova J, Raaka BM, Ghysdael J & Samuels HH 1992 Half-site spacing and orientation determines whether thyroid hormone and retinoic acid receptors and related factors bind to DNA response elements as monomers, homodimers, or heterodimers. *Molecular Endocrinology* **6** 429–442.
- Heery DM, Kalkhoven E, Hoare S & Parker MG 1997 A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature* **387** 733–736.
- Heinzel T, Lavinsky RM, Mullen T-M, Soderstrom M, Laherty CD, Torchia J, Yang W-M, Brard G, Ngo SD, Davie JR *et al.* 1997 A complex containing N-CoR, mSIN3 and histone deacetylase mediates transcriptional repression. *Nature* **387** 43–48.
- Horlein AJ, Naar AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Soderstrom M, Glass CK *et al.* 1995 Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature* **377** 397–404.
- Hu X & Lazar MA 1999 The CoNR motif controls the recruitment of corepressors by nuclear hormone receptors. *Nature* **402** 93–99.
- Kitajima K, Nagaya T & Jameson JL 1995 Dominant negative and DNA-binding properties of mutant thyroid hormone receptors that are defective in homodimerization but not heterodimerization. *Thyroid* **5** 343–353.
- Lazar MA, Berrodin TJ & Harding HP 1991 Differential DNA binding by monomeric, homodimeric, and potentially heteromeric forms of the thyroid hormone receptor. *Molecular and Cellular Biology* **11** 5005–5015.
- LeDourarin B, Zechel C, Garnier JM, Lutz Y, Tora L, Pierrat P, Heery D, Gronemeyer H, Chambon P & Losson R 1995 The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. *EMBO Journal* **14** 2020–2033.
- Lin KH, Willingham MC, Liang C-M & Cheng S-Y 1991 Intracellular distribution of the endogenous and transfected β form of thyroid hormone nuclear receptor visualized by the use of domain-specific monoclonal antibodies. *Endocrinology* **128** 2601–2609.
- Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P *et al.* 1995 The nuclear receptor superfamily: the second decade. *Cell* **83** 835–839.
- Marimuthu A, Feng W, Tagami T, Nguyen H, Jameson JL, Fletterick RJ, Baxter JD & West BL 2002 TR surfaces and conformations required to bind nuclear receptor corepressor. *Molecular Endocrinology* **16** 271–286.
- Nagaya T & Jameson JL 1993 Thyroid hormone receptor dimerization is required for dominant negative inhibition by mutations that cause thyroid hormone resistance. *Journal of Biological Chemistry* **268** 15766–15771.
- Nagy L, Kao HY, Chakravarti D, Lin RJ, Hassig CA, Ayer DE, Schreiber SL & Evans RM 1997 Nuclear receptor repression mediated by a complex containing SMRT, mSIN3 and histone deacetylase. *Cell* **89** 373–380.
- Nagy L, Kao H-Y, Love JD, Li C, Banayo E, Gooch JT, Chatterjee VKK, Evans RM & Schwabe JWR 1999 Mechanism of corepressor binding and release from nuclear hormone receptors. *Genes and Development* **13** 3209–3216.
- Nolte RT, Wisely GB, Westin S, Cobb JE, Lambert MH, Kurokawa R, Rosenfeld MG, Willson TM, Glass CK & Milburn MV 1998 Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor- γ . *Nature* **395** 137–143.
- Perissi V, Staszewski LM, Naar AM, McInerney EM, Kurokawa R, Kronen A, Rose DW, Lambert MH, Milburn MV, Glass CK *et al.* 1999 Molecular determinations of nuclear receptor-corepressor interaction. *Genes and Development* **13** 3198–3208.

- Pissios P, Trameli I, Kushner PJ & Moore DD 2000 Dynamic stabilization of nuclear receptor ligand binding domains by hormone or corepressor binding. *Molecular Cell* **6** 245–253.
- Rachez C, Suldan Z, Ward J, Chang C-P, Burakov D, Erdjument-Bromage H, Tempst P & Freedman LP 1998 A novel protein complex that interacts with the vitamin D3 receptor in a ligand-dependent manner and enhances VDR transactivation in a cell-free system. *Genes and Development* **12** 1787–1800.
- Refetoff S, Weiss RE & Usala SJ 1993 The syndromes of resistance to thyroid hormone. *Endocrine Reviews* **14** 348–399.
- Renaud JP, Rochel N, Ruff M, Vivat V, Chambon P, Gronemeyer H & Moras D 1995 Crystal structure of the RAR-gamma ligand-binding domain bound to all-trans retinoic acid. *Nature* **378** 681–689.
- Ribeiro RCJ, Feng W, Wagner RL, Costa CHRM, Pereira AC, Apriletti JW, Fletterick RJ & Baxter JD 2001 Definition of the surface in the thyroid hormone receptor ligand binding domain for association as homodimers and heterodimers with retinoid X receptor. *Journal of Biological Chemistry* **276** 14987–14995.
- Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA & Greene GL 1998 The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95** 927–937.
- Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA, McKenna NJ, Onate SA, Tsai SY, Tsai M-J *et al.* 1997 Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature* **389** 194–198.
- Tagami T & Jameson JL 1998 Nuclear corepressors enhance the dominant negative activity of mutant receptors that cause resistance to thyroid hormone. *Endocrinology* **139** 640–650.
- Tagami T, Nakamura H, Sasaki S, Miyoshi Y & Imura H 1993 Estimation of the protein content of thyroid hormone receptor α 1 and β 1 in rat tissues by Western blotting. *Endocrinology* **132** 275–279.
- Tagami T, Madison LD, Nagaya T & Jameson JL 1997a Nuclear receptor corepressors activate rather than suppress basal transcription of genes that are negatively regulated by thyroid hormone. *Molecular and Cellular Biology* **17** 2642–2648.
- Tagami T, Nakamura H, Sasaki S, Miyoshi Y & Nakao K 1997b Dimerization properties of mutant thyroid hormone β -receptors with auxiliary proteins. *Journal of Endocrinology* **154** 523–533.
- Tagami T, Lutz WH, Kumar R & Jameson JL 1998a The interaction of the vitamin D receptor with nuclear receptor corepressors and coactivators. *Biochemical and Biophysical Research Communications* **253** 358–363.
- Tagami T, Gu W, Peairs PT, West BL & Jameson JL 1998b A novel natural mutation in the thyroid hormone receptor defines a dual functional domain that exchanges nuclear receptor corepressors and coactivators. *Molecular Endocrinology* **12** 1888–1902.
- Tagami T, Kopp P, Johnson W, Arseven OK & Jameson JL 1998c The thyroid hormone receptor variant α 2 is a weak antagonist because it is deficient in interactions with nuclear receptor corepressor. *Endocrinology* **139** 2535–2544.
- Tagami T, Park Y & Jameson JL 1999 Mechanisms that mediate negative regulation of the thyroid-stimulating hormone α gene by the thyroid hormone receptor. *Journal of Biological Chemistry* **274** 22345–22353.
- Torchia J, Rose DW, Inostrova J, Kamei Y, Westin S, Glass CK & Rosenfeld MG 1997 The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* **387** 677–684.
- Umesono K, Murakami KK, Thompson CC & Evans RM 1991 Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D receptors. *Cell* **65** 1255–1266.
- Wagner RL, Apriletti JW, McGrath ME, West BL, Baxter JD & Fletterick RJ 1995 A structural role for hormone in the thyroid hormone receptor. *Nature* **378** 690–697.
- Webb P, Anderson CM, Valentine C, Nguyen P, Marimuthu A, West BL, Baxter JD & Kushner PJ 2000 The nuclear receptor corepressor (N-CoR) contains three isoleucine motifs (I/LXXII) that serve as receptor interaction domains (IDs). *Molecular Endocrinology* **14** 1976–1985.
- Yen PM, Darling DS, Carter RL, Forgione M, Umeda PK & Chin WW 1992 Triiodothyronine (T_3) binding decreases binding to DNA by T_3 -receptor homodimers but not receptor auxiliary protein heterodimers. *Journal of Biological Chemistry* **267** 3565–3568.
- Yoh SM, Chatterjee VKK & Privalsky ML 1997 Thyroid hormone resistance syndrome manifests as an aberrant interaction between mutant T_3 receptors and transcriptional corepressors. *Molecular Endocrinology* **11** 470–480.
- Zhang J, Zamir I & Lazar MA 1997 Differential recognition of liganded and unliganded thyroid hormone receptor by retinoid X receptor regulates transcriptional repression. *Molecular and Cellular Biology* **17** 6887–6897.

Received in final form 28 January 2009

Accepted 11 February 2009

Made available online as an Accepted Preprint 11 February 2009