Differences between the silencing-related properties of the extreme carboxyl-terminal regions of thyroid hormone receptors α1 and β1

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Abstract

Human thyroid hormone receptor (TR) is encoded by two distinct genes, TRα and TRβ. TR heterodimerizes with retinoid X receptor (RXR) and binds efficiently to the thyroid hormone (T3) response element (TRE) of target genes. In the absence of T3, unliganded TR suppresses the basal promoter activity of positively regulated genes (silencing). Silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor co-repressor (N-CoR) interact with unliganded TR and function as corepressor proteins. Previously, we found that TR deletion on a subset of TREs. In the present study, to assess the isoform-specific effects of the C-terminal truncations on TR silencing, we constructed two mutant TRα1s (αF397X and αE395X) with the same respective C-terminal truncations as βF451X and βE449X and analysed their silencing activities. Unlike βF451X and βE449X, αF397X and αE395X showed similarly stronger silencing potency than wild-type TRα1. We further studied the abilities of wild-type and the mutant TRβ1s and α1s on RXR and co-repressor binding by a two-hybrid interference assay. βF451X had significantly stronger abilities to bind to RXR and SMRT than did wild-type TRβ1 and βE449X. In contrast, wild-type TRα1, αF397X and αE395X showed similar abilities to bind to RXR and SMRT. βE449X and αE395X, which have identical C-terminal truncation, showed less ability to bind to N-CoR than did wild-type TRβ1 and βF451X and wild-type TRα1 and αF397X respectively. These results indicate that an identical C-terminal truncation gives rise to different effects on TRβ1 and α1 with respect to silencing potency, RXR binding and SMRT binding. The difference in the silencing potency among wild-type TRβ1, βF451X and βE449X correlated well with the difference in the ability to bind co-repressor SMRT.


Introduction

Thyroid hormone (T3) regulates target gene expression through T3-response elements (TREs), which bind thyroid hormone receptor (TR). TREs present in positively regulated elements are repeats of a half-site consensus motif (AGGTCA) oriented as a palindrome with no spacers (pal), a direct repeat with a 4-bp spacer (DR4) or an inverted palindrome with a 6-bp spacer (IP). High-affinity binding of TR to these TREs requires heterodimerization with retinoid X receptor (RXR) (Leid et al. 1992, Yu et al. 1992, Zhang et al. 1992). TR forms a TR homodimer, but TR homodimers have been found to be unstable on most TREs and are released from DNA in the presence of T3 (Yen et al. 1992). In mammals, TR is encoded by two distinct genes, TRα and TRβ, which produce two major isoforms, TRα1 and TRβ1 (Lazar 1993). Although TRα1 and β1 have the same binding affinity for T3 and the 46 amino acids in the extreme C-terminal region are completely identical between TRα1 and TRβ1, isoform specificity has been demonstrated on certain TREs. TRα1 is more potent as a ligand-dependent activator on certain positive TREs (Hollenberg et al. 1995). In contrast, TRβ1 is twofold more potent than TRα1 on TSHα subunit gene as a ligand-dependent silencer (McCabe et al. 1998). Besides its ligand-dependent properties, TR functions as a ligand-independent silencer on positive TREs (Brent et al. 1989, Zhang et al. 1991) and as a ligand-independent activator on negatively regulated genes (Flynn et al. 1994, Tagami & Jameson 1998). Co-repressor proteins such as silencing mediator for retinoid and thyroid hormone receptors (SMRT; Chen & Evans 1995) and nuclear receptor co-repressor (N-CoR; Hörlein et al. 1995) are involved in unliganded TR-mediated transcriptional repression on positive TREs (silencing).

We have previously identified two different mutant TRβ1s that have a C-terminal 11-amino acid (βF451X)
and 13-amino acid (βE449X) truncation in patients with resistance to thyroid hormone (RTH; Miyoshi et al. 1995, 1998). Although βF451X and βE449X have the same functional disruption of the autonomous activation function domain (AF2) and negligible T₃-binding activity, the silencing potency of βF451X was stronger than that of βE449X and wild-type TRβ1 (Miyoshi et al. 1998). We also analysed the DNA-binding and dimerization properties of wild-type TRβ1 and βF451X by a gel-shift assay, but no apparent difference was observed in DNA binding between wild-type TRβ1 and βF451X (Nishiyama et al. 1998).

The TR–RXR heterodimer is responsible for the silencing, as TR mutants that are defective in their ability to homodimerize but still have the ability to heterodimerize with RXR retain their ability to act as transcriptional silencers (Flynn et al. 1994). In addition, TRα1 amino (N)-terminus antagonizes homodimer formation on positive TREs and deletion of the N-terminus from TRα1 leads to preferential homodimer formation and decreased silencing activity, suggesting the possibility that enhanced homodimerization would lead to less silencing (Hollenberg et al. 1995). The association with corepressor proteins is crucial for the TR-mediated silencing effect (Yen et al. 1995). A strong positive correlation was found between mutant TR interactions with co-repressor N-CoR and transcriptional silencing activity (Tagami & Jameson 1998). The AF2 domain in the extreme C-terminal region of TR has a crucial role for not only T₃-dependent transactivation but also T₃-induced release of co-repressor proteins (Barettoni et al. 1994, Baniahmad et al. 1995).

Thus in the present study, to analyse the silencing effects mediated by the C-terminal truncated TRs, we have focused our attention on the association of mutant TRs with RXR and co-repressor proteins.

To identify whether or not the same C-terminal truncations as βF451X and βE449X exert similar effects on TRα1 with respect to TR silencing, we artificially constructed two mutant TRα1s (αF397X and αE395X) with C-terminal truncations identical to those in βF451X and βE449X respectively and analysed the silencing effects of the truncated TRα1s. We examined the abilities of the TRα1s and β1s to bind RXR, co-repressor SMRT and co-repressor N-CoR by a two-hybrid interference assay. We demonstrate here the isoform-specific effects of the C-terminal truncations on TR silencing and clarify the relationship between the silencing potency and the abilities to bind to RXR and co-repressor proteins.

Materials and Methods

Plasmids

A reporter plasmid (GalpX3-tk-Luc) that contains a Gal4 response element upstream of the luciferase gene, expression vectors (pCMX) encoding fusion proteins Gal4 RXRαLBD and Gal4 SMRT in which the hinge region and the ligand-binding domain (H-LBD) of human RXRα and the interacting domain (ID) of human co-repressor SMRT were fused to the C-terminus of the DNA-binding domain (DBD) of yeast nuclear transcriptional factor Gal4 respectively, and a pCMX for a fusion protein VP16TRβ1 LBD in which the activation domain of herpes simplex virus transcriptional factor VP16 was linked to the N-terminus of H-LBD of human TRβ1 (residues 174–461) were gifts from Dr K Umesono (Kyoto University, Japan). A cDNA for human N-CoR (Hollenberg et al. 1996) was digested with EcoRI and the resulting EcoRI–EcoRI fragment that encodes N-CoR ID was inserted downstream of the cDNA for Gal4 DBD in pCMX to produce a fusion protein, Gal4N-CoR. A pCMX for βE449X was constructed by substituting a DNA fragment spanning the base substitutions for the identical portion of wild-type TRβ1 cDNA. A pCMX encoding αE395X with a C-terminal truncation identical to that in βE449X was created by inserting a stop codon immediately after codon 394 of human TRα1 as follows. Polymerase chain reaction (PCR) was performed with a set of primers: a forward primer corresponding to nucleotides 1097–1128 of human TRα1 cDNA, 5’-GGAGG CTGACCCTGCTGGCGTTCGAGCACTAC-3’, which has a BspMI recognition site; a reverse primer corresponding to nucleotides 1247–1289, 5’-TCAAGACCTCTGAGGAAGAGTGGGGGAAGAGTTAGGTTGGGCC-3’, which has base substitutions to introduce a stop codon immediately 3’ to codon 394 and an Xhol recognition sequence downstream of the inserted stop codon. The PCR product was digested with BspMI and XhoI and the resulting 161-bp DNA fragment was substituted for the identical portion of wild-type human TRα1 cDNA. The base substitutions in the recombinant expression vectors were confirmed by DNA sequencing. Expression vectors (pCMX) encoding human TRβ1, TRα1, βF451X, αF397X and human RXRα were the same as those used in the reference reported by Nishiyama et al. (1998). Reporter plasmids containing TRE-DR4, TRE-pal2 and the chicken lysozyme silencer F2-TRE (F2-TRE) were described previously (Nishiyama et al. 1998).

pCMXs expressing N-terminally epitope (FLAG)-fused wild-type and mutant TRs were constructed by inserting a synthesized double-strand DNA encoding FLAG peptide (DYKDDDDK) upstream of the coding region of the TRβ1s and the TRα1s. An Asp718 recognition site was created immediately upstream of the coding region of the TRs by PCR. A DNA encoding the FLAG peptide was made by annealing the oligonucleotides 5’-AGCTTGAA TTCCACCATGGACTACAAGGACGACGATGACA AGG-3’ and 5’-GTACCCCTTGTCATCGTCGCCCT GTATGCCCATGGTTGAAATTCA-3’, and the resulting duplex was inserted into the HindIII-Asp718 site of reporter plasmids encoding wild-type and mutant TRβ1s and TRα1s. These expression vectors encode fusion
proteins that begin with an initiation Met and FLAG peptide, followed by amino acids of the wild-type and mutant TRβ1s and TRα1s. As a result of subcloning, a stretch of two amino acids (Gly, Val) was inserted between the FLAG peptide and the TR coding region.

Cell culture, transient transfection and silencing assay
CV-1 is an established cell line derived from African green monkey kidney and the cells express RXR, SMRT and N-CoR endogenously, but do not produce any isoforms of endogenous TR (Misiti et al. 1998). CV-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% thyroid hormone-depleted fetal calf serum (FCS) (Samuels et al. 1979), 100 units/ml penicillin G and 100 µg/ml streptomycin in 60 mm dishes. After the cells reached a density of 10⁶ cells/dish, they were transfected with 0·05 pmol/dish of empty expression vector or pCMX encoding wild-type or mutant TR along with 1·8 µg/dish of a reporter plasmid and 3·6 µg/dish of β-galactosidase expression vector pCH111 (Pharmacia LKB Biotechnology, Piscataway, NJ, USA) by calcium phosphate precipitation (Umesono et al. 1991). Bluescript-SK(+) (Stratagene, La Jolla, CA, USA) was used as carrier DNA to adjust the total amount of DNA to 7·2 µg/dish. After exposure to the calcium phosphate–DNA precipitate for 20 h, the cells were washed with PBS and incubated in DMEM with 5% thyroid hormone-depleted FCS for an additional 24 h for the chloramphenicol acetyltransferase (CAT) assay or 48 h for the luciferase assay. CAT and luciferase activities were measured according to the methods of Neumann et al. (1987) and De Wet et al. (1987) respectively. The transfection efficiency was normalized by the β-galactosidase activity (Herbomel et al. 1984). The silencing effect of TR was analysed by comparing the reporter gene activities obtained in the transfections with empty expression vector and TR expression vector.

Mammalian two-hybrid interference assay
The expression plasmids (0·05 pmol/dish each) for Gal4–fusion proteins (Gal4 RXRαLBD, Gal4 SMRT and Gal4N-CoR) and VP16TRβ1 LBD were transfected into CV-1 cells along with 0·05 pmol/dish of empty expression vector pCMX or pCMX encoding wild-type or mutant TR, except that 0·0125 pmol/dish of VP16TRβ1 LBD was transfected in the examination of the interaction of TR with co-repressor N-CoR. Luciferase reporter plasmid Galp33-tk-Luc (1·8 µg/dish) and β-galactosidase expression vector pCH111 (3·6 µg/dish) were also cotransfected into the cells. The abilities of wild-type and mutant TRs to bind RXRα, SMRT and N-CoR were compared by measuring the competitive inhibition of the VP16-induced Gal4-reporter gene activity by a coexpressed wild-type or mutant TR. The transfection efficiency was normalized by the β-galactosidase activity (Herbomel et al. 1984).

Immunoblot analysis
CV-1 cells in 60 mm dishes were transfected with 1 pmol/dish of empty expression vector (pCMX) or pCMX encoding FLAG-fused wild-type or mutant TR along with 3·6 µg/dish of β-galactosidase expression vector pCH111, and incubated in DMEM with 5% thyroid hormone-depleted FCS for 48 h. The cells were scraped in 150 µl of lysis buffer consisting of 125 mM Tris-HCl (pH 7·5) and 0·5% Triton X-100, and then centrifuged at 17 500 g for 5 min at 4 °C. The resulting supernatant was assayed for β-galactosidase activity to normalize for the transfection efficiency. The cell pellet, including nuclei, was dissolved in SDS-PAGE loading buffer consisting of 62·5 mM Tris-HCl (pH 7·5), 2·3% SDS, 0·64 M β-mercaptoethanol and 10% glycerol, and the proteins in an equivalent amount of each nuclear lysate were separated by SDS-10%PAGE and blotted onto a PVDF membrane (Micro Separations Inc., Westboro, MA, USA). Incubations with the primary anti-FLAG M2 monoclonal antibody (Sigma Chemical Co., St Louis, MO, USA) and with the secondary horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (Amersham International plc, Amersham, Bucks, UK) were carried out according to the manufacturer’s instructions. The membrane was incubated with 15 ml 50 mM Tris-HCl (pH 7·6) buffer containing 4 mg 3,3′-diaminobenzidine and 0·01% hydrogen peroxide for 10 min at room temperature to develop the colouring reaction.

Statistical analysis
The significance of differences in CAT and luciferase activities was determined by ANOVA and Fisher’s PLSD test using StatView 4·0 (Abacus Concepts, Berkeley, CA, USA).

Results
Silencing effects of wild-type and mutant TRβ1s
Silencing activities of wild-type and mutant TRβ1s were determined on various TREs by transiently transfecting each of the TRβ1-expression vectors into CV-1 cells. On TRE-DR4, wild-type TRβ1, βF451X and βE449X suppressed the basal promoter activity to 56·0 ± 12·3, 34·3 ± 6·8 and 50·5 ± 12·5% relative to the activity without TR coexpression respectively (Fig. 1A). βF451X showed a significantly stronger silencing effect than wild-type TRβ1 and βE449X. There was no significant difference in silencing activity between wild-type TRβ1 and βE449X on TRE-DR4. On TRE-pal2, βF451X also
exhibited a significantly stronger silencing effect than wild-type TRβ1 and βE449X (Fig. 1B). The silencing effects of wild-type TRβ1 and βE449X were equivalent on TRE-pal2. On the F2-TRE, βF451X showed a significantly stronger silencing effect than βE449X, but no significant difference was observed between βF451X and wild-type TRβ1 (Fig. 1C). The silencing effects of wild-type and mutant TRs on the F2-TRE were weaker than those on TRE-DR4 and TRE-pal2.

Silencing effects of wild-type and mutant TRα1s
The silencing activities of wild-type and mutant TRα1s were analysed in experiments together with the TRβ1s. On TRE-DR4, αF397X and αE395X suppressed the basal promoter activity to 59.3 ± 7.7, 28.4 ± 4.1 and 31.7 ± 6.7% relative to the activity without TR co-expression respectively (Fig. 2A). αF397X and αE395X showed similar and significantly stronger silencing activities than wild-type TRα1. On TRE-pal2, αF397X and αE395X also showed similar and significantly stronger silencing effects than wild-type TRα1 (Fig. 2B). On the F2-TRE, the silencing activities of wild-type TRα1, αF397X and αE395X did not differ significantly (Fig. 2C). The silencing effects of αF397X and αE395X on the F2-TRE were considerably weaker than those on TRE-DR4 and -pal2.

Association of TR with RXR, SMRT and N-CoR
The abilities of wild-type and mutant TRs to associate with human RXRα, co-repressor SMRT and N-CoR in
solution were analysed by a mammalian two-hybrid interference assay. Wild-type and mutant TRs were coexpressed with the two hybrid proteins, VP16-TRβ1 LBD and one of the Gal4 DBD-fused proteins (Gal4 RXRαLBD, Gal4 SMRT or Gal4N-CoR) in CV-1 cells. The full-length TRs competitively inhibited the interaction between VP16-TRβ1 LBD and the Gal4 DBD-fused proteins. βF451X had significantly greater ability to associate with RXR than wild-type TRβ1 and βE449X (Fig. 3A). βF451X also showed significantly greater ability to associate with SMRT than wild-type TRβ1 and βE449X (Fig. 3B). Wild-type TRβ1 and βE449X exhibited similar abilities to bind to SMRT. No difference was observed between the abilities of wild-type TRβ1 and βF451X to bind to N-CoR, but βE449X showed significantly less ability to associate with N-CoR (Fig. 3C).

In contrast to the wild-type and mutant TRβ1s, no significant difference was observed among wild-type TRα1, αF397X and αE395X in the abilities to associate with RXR and SMRT (Fig. 4A and B). αE395X had significantly less ability than wild-type TRα1 and αF397X to associate with N-CoR (Fig. 4C).

**Immunoblot analysis of transfected TRs**

To exclude the possibility of marked differences in the levels of TR proteins expressed, we transfected plasmids encoding FLAG-fused TRs into CV-1 cells and estimated the amounts of the TRs by immunoblot analysis. The anti-FLAG monoclonal antibody successfully detected the N-terminally located FLAG sequence in the TR proteins (Fig. 5). No remarkable difference was observed in the density of the FLAG-fused TR bands among wild-type
Discussion

In this study we analysed isoform-specific roles of the AF2 domain in TR silencing by expressing wild-type and variously C-terminally truncated mutant TRβ1s and α1s in CV-1 cells. The silencing effects were examined on three different positive TREs: TRE-DR4, TRE-pal2 and the F2-TRE. Because it is uncertain that VP16-coupled TR possesses the same properties as untagged TR on the interaction with other proteins, we did not simply assay the interaction of Gal4-fusion proteins with VP16-coupled wild-type or mutant TRs but chose a two-hybrid interference assay to analyse the ability of each TR to bind RXR and co-repressor proteins. The immunoblot analysis (Fig. 5) indicates that the transfected CV-1 cells expressed wild-type and the mutant TRs to an equal level.

The silencing effects on the F2-TRE were relatively weak (Figs 1C, 2C). The reason for the weak silencing is not clear, but might be related to the fact that TR homodimers bind more efficiently to the F2-TRE (Yen et al. 1992). βF451X exhibited stronger silencing effects than wild-type TRβ1 and βE449X on TRE-DR4 and
TRE-pal2, which bound TR-RXR heterodimers more efficiently than TR homodimers (Wahlström et al. 1992, Zhang et al. 1992) (Fig. 1). In parallel with the stronger silencing effects, βF451X had greater ability to associate with RXR and co-repressor SMRT (Fig. 3). The stronger silencing potency of βF451X was believed to be, at least in part, due to its increased ability to interact with RXR and SMRT. It has been reported that RXR potentiates and stabilizes TR–co-repressor interaction in solution (Zhang et al. 1997). However, βF451X did not increase the ability of TR to associate with N-CoR, despite the enhanced heterodimerization with RXR (Fig. 3A and C). The silencing potency of βE449X was equal to that of wild-type TRβ1, consistent with their similar recruitment of co-repressor SMRT (Figs 1, 3B). These results indicate that the silencing potencies of wild-type TRβ1, βF451X and βE449X on positive TREs are correlated with the ability to bind SMRT, not RXR or N-CoR.

Conversely, αF397X and αE395X, which have the same C-terminal truncations as βF451X and βE449X, respectively, exhibited equivalently stronger silencing effects than wild-type TRα1 on TRE-DR4 and TRE-pal2 (Fig. 2). This indicates that the identical truncations in the extreme C-terminal regions of TRβ1 and α1 produce different effects on TR silencing. Regarding the association with RXR and co-repressor proteins, in contrast to the TRβ1s, no significant difference was observed in the associations with RXR and SMRT among the wild-type and mutant TRα1s (Fig. 4A and B). This result also indicates that the identical C-terminal truncations in TRβ1 and α1 give rise to isoform-specific effects on the interaction with RXR and SMRT.

Figure 4 RXR and co-repressor binding with wild-type and mutant TRα1s analysed by mammalian two-hybrid interference assay. The experimental procedures are described in Materials and Methods. The luciferase activity determined by cotransfecting empty expression vector pCMX was expressed as 100% ( []). The activities resulting from cotransfection of wild-type TRα1 (stippled column), αF397X (hatched column) or αE395X (■) were expressed relative to that resulting from cotransfection with empty expression vector pCMX. (A) Interaction with RXR. (B) Interaction with co-repressor SMRT. (C) Interaction with co-repressor N-CoR. The data were expressed as mean ± s.d. of four (A), seven (B) and four (C) separate assays. *Significant difference (P<0.05).
co-repressor SMRT. Unlike the effect on SMRT-binding, the identical C-terminal truncations in TRβ1 and α caused similar effects on the interaction with N-CoR (Figs 2C, 4C). Although αE395X had less ability to interact with N-CoR than did wild-type TRα1 and αF397X, αE395X still retained a stronger silencing activity than wild-type TRα1 and αF397X on TRE-DR4 and -pal2. The silencing potency of wild-type TRα1, αF397X and αE395X was not correlated with the ability to bind RXR, SMRT or N-CoR, suggesting that the mechanism of TRα1-mediated silencing on positive TRES may be different from that of TRβ1.

Because both positive regulation and negative-feedback regulation by T3 are disrupted (Refetoff et al. 1993) in patients with RTH, it is interesting to study the dominant negative effects of RTH mutants on negatively regulated genes, such as TSH-β and α subunits. In contrast to the rapid progress in the area of T3-dependent and independent regulation on positive TRES, the mechanism of T3-mediated transcriptional regulation on negatively regulated genes remains unclear. Sasaki et al. (1999) reported that T3-dependent histone deacetylase (HDAC) recruitment is a mechanism for negative feedback regulation of the TSH-β subunit gene. They demonstrated that liganded TR interacted with and recruited HDAC2 to a negative regulatory element of the TSH-β subunit gene without RXR and co-repressor proteins, raising the possibility that co-repressors are not involved in HDAC recruitment to the negative regulatory element. In contrast, basal promoter activation by unliganded TRβ1 was abolished by the mutation that impairs co-repressor interaction on the TSH-α subunit gene (Tagami et al. 1999). That result supports the idea that co-repressor proteins are involved in the regulation of genes that are negatively regulated by T3. Furthermore, wild-type TRβ1 with greater binding affinity for the TSH-α subunit gene was more potent in T3-dependent repression than was wild-type TRα1 (McCabe et al. 1998). These findings suggest the possibility that differential HDAC recruitment, co-repressor association and DNA binding may determine the ability of wild-type and mutant TRs to regulate transcription on negatively regulated genes. Very recently, we have found that negative regulation of the TSH-β gene by T3/α can be observed even in CV-1 cells when they are exogenously expressed pituitary transcriptional activator-1 and GATA transcriptional factor-2. Using this system, we are now studying dominant negative effects of the C-terminal-truncated mutant TRα1s and β1s on the TSH-β subunit gene promoter.

In summary, we have clarified that identical truncations in the extreme C-terminal region give rise to isoform specific-effects on TRβ1 and TRα1 with respect to silencing activity and interactions with RXR and co-repressor SMRT. On the basis of the analysis of the relationship between the silencing potency and the abilities to bind RXR, SMRT and N-CoR, we suppose that the superior association with RXR and SMRT might be one of the mechanisms for the strong silencing activity of BF451X. The silencing potency among wild-type TRβ1, BF451X and BE449X were well correlated to the ability to bind co-repressor SMRT.

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