Differences between the silencing-related properties of the extreme carboxyl-terminal regions of thyroid hormone receptors $\alpha 1$ and $\beta 1$

K Nishiyama, A Matsushita, H Natsume, T Mikami, R Genma, S Sasaki and H Nakamura

Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu, Shizuoka 431-3192, Japan
(Requests for offprints should be addressed to K Nishiyama, Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine, 3600 Handa-Cho, Hamamatsu, Shizuoka 431-3192, Japan)

Abstract

Human thyroid hormone receptor (TR) is encoded by two distinct genes, TR α and TR β . TR heterodimerizes with retinoid X receptor (RXR) and binds efficiently to the thyroid hormone (T_3) response element (TRE) of target genes. In the absence of T_3 , 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 $\beta F451X$ with carboxyl (C)-terminal 11-amino acid deletion had stronger silencing potency than wild-type TR $\beta 1$ and $\beta E449X$ with C-terminal 13-amino acid 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 TR $\alpha 1$ s ($\alpha F397X$ and $\alpha E395X$) with the same respective C-terminal truncations as $\beta F451X$ and $\beta E449X$ and analysed their silencing activities. Unlike $\beta F451X$ and

$\beta E449X$, $\alpha F397X$ and $\alpha E395X$ showed similarly stronger silencing potency than wild-type TR $\alpha 1$. We further studied the abilities of wild-type and the mutant TR $\beta 1$ s and $\alpha 1$ s on RXR and co-repressor binding by a two-hybrid interference assay. $\beta F451X$ had significantly stronger abilities to bind to RXR and SMRT than did wild-type TR $\beta 1$ and $\beta E449X$. In contrast, wild-type TR $\alpha 1$, $\alpha F397X$ and $\alpha E395X$ showed similar abilities to bind to RXR and SMRT. $\beta E449X$ and $\alpha E395X$, which have identical C-terminal truncation, showed less ability to bind to N-CoR than did wild-type TR $\beta 1$ and $\beta F451X$ and wild-type TR $\alpha 1$ and $\alpha F397X$ respectively. These results indicate that an identical C-terminal truncation gives rise to different effects on TR $\beta 1$ and $\alpha 1$ with respect to silencing potency, RXR binding and SMRT binding. The difference in the silencing potency among wild-type TR $\beta 1$, $\beta F451X$ and $\beta E449X$ correlated well with the difference in the ability to bind co-repressor SMRT.

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Introduction

Thyroid hormone (T_3) regulates target gene expression through T_3 -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 T_3 (Yen *et al.* 1992). In mammals, TR is encoded by two distinct genes, TR α and TR β , which produce two major isoforms, TR $\alpha 1$ and TR $\beta 1$ (Lazar 1993). Although TR $\alpha 1$ and $\beta 1$ have the same binding affinity for T_3 and the 46 amino acids in the extreme

C-terminal region are completely identical between TR $\alpha 1$ and TR $\beta 1$, isoform specificity has been demonstrated on certain TREs. TR $\alpha 1$ is more potent as a ligand-dependent activator on certain positive TREs (Hollenberg *et al.* 1995). In contrast, TR $\beta 1$ is twofold more potent than TR $\alpha 1$ on TSH α 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 TR $\beta 1$ s that have a C-terminal 11-amino acid ($\beta F451X$)

and 13-amino acid (β E449X) truncation in patients with resistance to thyroid hormone (RTH; Miyoshi *et al.* 1995, 1998). Although β F451X and β E449X have the same functional disruption of the autonomous activation function domain (AF2) and negligible T_3 -binding activity, the silencing potency of β F451X was stronger than that of β E449X and wild-type TR β 1 (Miyoshi *et al.* 1998). We also analysed the DNA-binding and dimerization properties of wild-type TR β 1 and β F451X by a gel-shift assay, but no apparent difference was observed in DNA binding between wild-type TR β 1 and β F451X (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, TR α 1 amino (N)-terminus antagonizes homodimer formation on positive TREs and deletion of the N-terminus from TR α 1 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 co-repressor 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 T_3 -dependent transactivation but also T_3 -induced release of co-repressor proteins (Baretino *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 β F451X and β E449X exert similar effects on TR α 1 with respect to TR silencing, we artificially constructed two mutant TR α 1s (α F397X and α E395X) with C-terminal truncations identical to those in β F451X and β E449X respectively and analysed the silencing effects of the truncated TR α 1s. We examined the abilities of the TR α 1s and β 1s 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

RXR α LBD and Gal4 SMRT in which the hinge region and the ligand-binding domain (H-LBD) of human RXR α 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 VP16TR β 1 LBD in which the activation domain of herpes simplex virus transcriptional factor VP16 was linked to the N-terminus of H-LBD of human TR β 1 (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 β E449X was constructed by substituting a DNA fragment spanning the base substitutions for the identical portion of wild-type TR β 1 cDNA. A pCMX encoding α E395X with a C-terminal truncation identical to that in β E449X was created by inserting a stop codon immediately after codon 394 of human TR α 1 as follows. Polymerase chain reaction (PCR) was performed with a set of primers: a forward primer corresponding to nucleotides 1097–1128 of human TR α 1 cDNA, 5'-GGAGG CGTACCTGCTGGCGTTCGAGCACTAC-3', which has a BspMI recognition site; a reverse primer corresponding to nucleotides 1247–1289, 5'-TCAAAGACCTCGA GGAAGAGTGGGGGGAAGAGTTAGGTGGGGC-3', which has base substitutions to introduce a stop codon immediately 3' to codon 394 and an XhoI 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 TR α 1 cDNA. The base substitutions in the recombinant expression vectors were confirmed by DNA sequencing. Expression vectors (pCMX) encoding human TR β 1, TR α 1, β F451X, α F397X and human RXR α 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 TR β 1s and the TR α 1s. 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'-GTACCCTTGTCATCGTCGTCCTT GTAGTCCATGGTGGGAATTCA-3', and the resulting duplex was inserted into the HindIII-Asp718 site of pCMX vectors encoding wild-type and mutant TR β 1s and TR α 1s. 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 TR β 1s and TR α 1s. 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 μ g/ml streptomycin in 60 mm dishes. After the cells reached a density of 10^6 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 μ g/dish of a reporter plasmid and 3.6 μ g/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 μ g/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 RXR α LBD, Gal4 SMRT and Gal4N-CoR) and VP16TR β 1 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 VP16TR β 1 LBD was transfected in the examination of the interaction of TR with co-repressor N-CoR. Luciferase reporter plasmid Galpx3-tk-Luc (1.8 μ g/dish) and β -galactosidase expression vector pCH111 (3.6 μ g/dish) were also cotransfected into the cells. The abilities of wild-type and mutant TRs to bind RXR α , 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 μ g/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 μ l 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 TR β 1s

Silencing activities of wild-type and mutant TR β 1s were determined on various TREs by transiently transfecting each of the TR β 1-expression vectors into CV-1 cells. On TRE-DR4, wild-type TR β 1, β F451X and β E449X suppressed the basal promoter activity to 56.0 ± 12.3 , 34.3 ± 6.8 and $50.5 \pm 12.5\%$ relative to the activity without TR coexpression respectively (Fig. 1A). β F451X showed a significantly stronger silencing effect than wild-type TR β 1 and β E449X. There was no significant difference in silencing activity between wild-type TR β 1 and β E449X on TRE-pal2, β F451X also

Figure 1 Silencing effects of wild-type TRβ1, βF451X and βE449X on TRE-DR4 (A), TRE-pal2 (B) and the F2-TRE (C). Reporter gene activities obtained from the transfection with empty expression vector pCMX are expressed as 100% (□). The activities determined with wild-type TRβ1 (stippled columns), βF451X (hatched columns) and βE449X (■) are expressed relative to that with empty expression vector pCMX. The data are expressed as mean ± S.D. of six (TRE-DR4), eight (TRE-pal2) and five (F2-TRE) separate experiments. *Significant difference ($P < 0.05$).

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

$31.7 \pm 6.7\%$ 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

Figure 2 Silencing effects of wild-type TRα1, αF397X and αE395X on TRE-DR4 (A), TRE-pal2 (B) and the F2-TRE (C). Reporter gene activities obtained from the transfection with empty expression vector pCMX are expressed as 100% (□). The activities determined with wild-type TRα1 (stippled columns), αF397X (hatched columns) and αE395X (■) are expressed relative to that with empty expression vector pCMX. The data are expressed as mean ± s.d. of four independent experiments (on all the TREs). *Significant difference ($P<0.05$).

solution were analysed by a mammalian two-hybrid interference assay. Wild-type and mutant TRs were coexpressed with the two hybrid proteins, VP16TRβ1 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

Figure 3 RXR and co-repressor binding with wild-type and mutant TRβ1s 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 TRβ1 (stippled column), βF451X (hatched column) or βE449X (■) 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 ($P < 0.05$).

TRβ1, βF451X and βE449X and among wild-type TRα1, αF397X and αE395X. In the lanes for the wild-type and mutant TRβ1s, two additional bands with smaller molecular mass were found (asterisks in Fig. 5). These were assumed to be degradation products of the TRβ1s, and there was again no apparent difference in the density of the faster-migrating bands among wild-type and the C-terminal-truncated TRβ1s.

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

Figure 4 RXR and co-repressor binding with wild-type and mutant TR α 1s 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 TR α 1 (stippled column), α F397X (hatched column) or α E395X (■) 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 $\pm$ s.d. of four (A), seven (B) and four (C) separate assays. *Significant difference ($P < 0.05$).

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, β F451X had greater ability to associate with RXR and co-repressor SMRT (Fig. 3). The stronger silencing potency of β F451X 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, β F451X 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 β E449X was equal to that of wild-type TR β 1, consistent with their similar recruitment of co-repressor SMRT (Figs 1, 3B). These results indicate that the silencing potencies of wild-type TR β 1, β F451X

and β E449X on positive TREs are correlated with the ability to bind SMRT, not RXR or N-CoR.

Conversely, α F397X and α E395X, which have the same C-terminal truncations as β F451X and β E449X, respectively, exhibited equivalently stronger silencing effects than wild-type TR α 1 on TRE-DR4 and TRE-pal2 (Fig. 2). This indicates that the identical truncations in the extreme C-terminal regions of TR β 1 and α 1 produce different effects on TR silencing. Regarding the association with RXR and co-repressor proteins, in contrast to the TR β 1s, no significant difference was observed in the associations with RXR and SMRT among the wild-type and mutant TR α 1s (Fig. 4A and B). This result also indicates that the identical C-terminal truncations in TR β 1 and α 1 give rise to isoform-specific effects on the interaction with RXR and

Figure 5 Immunoblot analysis. The experimental procedures are described in Materials and Methods. Twenty microlitre aliquots of each nuclear lysate were subjected to the analysis. CV-1 cells transfected with pCMX (lane 1), pCMX-FLAGTR β 1 (lane 2), pCMX-FLAG β F451X (lane 3), pCMX-FLAG β E449X (lane 4), pCMX-FLAGTR α 1 (lane 5), pCMX-FLAG α F397X (lane 6) and pCMX-FLAG α E395X (lane 7) were analysed by SDS-10%PAGE. The molecular size markers were myosin (220 kDa), phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (21.5 kDa) from Amersham Life Science. *Assumed degradation products of the TR β 1s.

co-repressor SMRT. Unlike the effect on SMRT-binding, the identical C-terminal truncations in TR β 1 and α 1 caused similar effects on the interaction with N-CoR (Figs 2C, 4C). Although α E395X had less ability to interact with N-CoR than did wild-type TR α 1 and α F397X, α E395X still retained a stronger silencing activity than wild-type TR α 1 and α F397X on TRE-DR4 and -pal2. The silencing potency of wild-type TR α 1, α F397X and α E395X was not correlated with the ability to bind RXR, SMRT or N-CoR, suggesting that the mechanism of TR α 1-mediated silencing on positive TREs may be different from that of TR β 1.

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 TR-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 TR β 1 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 TR β 1 with greater binding affinity for the TSH- α subunit gene was more potent in T₃-dependent repression than was wild-type TR α 1 (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/TR 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 TR α 1s and β 1s 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 TR β 1 and TR α 1 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 β F451X. The silencing potency among wild-type TR β 1, β F451X and β E449X were well correlated to the ability to bind co-repressor SMRT.

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